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PERTANIKA JOURNAL OF TROPICAL AGRICULTURAL SCIENCE

About the Journal

Overview

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

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

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

The journal is available world-wide.

Aims and scope

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

History

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

Vision

To publish journals of international repute.

Mission

Our goal is to bring the highest quality research to the widest possible audience.

Quality

We aim for excellence, sustained by a responsible and professional approach to journal publishing. Submissions are guaranteed to receive a decision within 90 days. The elapsed time from submission to publication for the articles averages 180 days. We are working towards decreasing the processing time with the help of our editors and the reviewers.

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

Publication policy

Pertanika policy prohibits an author from submitting the same manuscript for concurrent consideration by two or more publications. It prohibits as well publication of any manuscript that has already been published either in whole or substantial part elsewhere. It also does not permit publication of manuscript that has been published in full in proceedings.

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A decision on acceptance or rejection of a manuscript is expected within 90 days (average). The elapsed time from submission to publication for the articles averages 180 days.

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Pertanika follows a double**-blind peer review** process. Manuscripts deemed suitable for publication are sent to reviewers. Authors are encouraged to suggest names of at least 3 potential reviewers at the time of submission of their manuscripts to *Pertanika,* but the editors will make the final selection and are not, however, bound by these suggestions.

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.

The journal's peer review

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:

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

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

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

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

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

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

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

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

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

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AN INTERNATIONAL PEER-REVIEWED JOURNAL

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Foreword

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

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

This issue contains 17 articles: two review articles; one short communication; and the rest are regular articles. The authors of these articles come from different countries namely Indonesia, Malaysia, Nigeria and Philippines.

A selected article entitled "Assessing Reproductive Ecology and Oviposition Habitat Selection Among Anurans in Ayer Hitam Forest Reserve, Puchong, Selangor" investigated the relationship between habitat characteristics and breeding site preferences of frogs and toads (anurans) in Compartment 15 of the Ayer Hitam Forest Reserve (AHFR). The study was conducted in both natural and artificial ponds. A total of 67 individuals from 18 species and six families were recorded. *Hylarana labialis* was the most abundant species near natural ponds, while *Kalophrynus palmatissimus*, an endangered and endemic species, was equally represented in both natural and artificial ponds. The findings emphasize how microclimate and habitat variables influence breeding success, and suggest that while certain species show resilience to disturbance, the long-term effects of habitat loss require further evaluation. The detailed information of this article is available on page 1497.

A study by Rina Ekawati and team entitled "Organic Fertilizer Formulation To Improve The Quality and Yield of *Eleutherine palmifolia* L. (Merr) As An Alley Cropping In Coffee Plantation" examined how different combinations of organic fertilizers affect the growth performance and medicinal quality of *E. palmifolia* cultivated under coffee tree shade. Results showed that the complete fertilization treatment (cow manure + guano + rice husk ash) led to significant improvements in growth parameters such as leaf production, shoot and root biomass, tuber yield per plant and per plot, as well as higher flavonoid content in bulbs compared to unfertilized controls. The study provides new evidence supporting the feasibility of intercropping *E. palmifolia* with coffee trees while enhancing both yield and phytochemical properties. Full information on this study is presented on page 1529.

A regular article entitled "Foliar Sprays of Biostimulants and PGRs Improve the Reproductive Parameters of Cacao Under Water Stress" explored the effectiveness of biostimulants (oligocarrageenan and oligochitosan) and plant growth regulators (cytokinin and paclobutrazol) as foliar sprays to enhance pod retention, bean quality, and overall yield of a six-year-old grafted cacao cultivar 'UF 18'. Treatments were applied under irrigated and rainfed conditions during the dry season. The results showed that oligochitosan increased flowering intensity, paclobutrazol reduced cherelle wilt incidence, and cytokinin enhanced pod retention. These treatments also improved physiological and yield traits such as leaf chlorophyll content, seed fresh weight, percentage of full beans, and bean count per tree. This study underscores the potential of biostimulant and PGR foliar sprays as practical interventions for sustaining cacao yield under water stress conditions. Further details of this study are found on page 1653.

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.

Editor-in-Chief
Mohamed Thariq Hameed Sultan

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Review Article

A Comprehensive Documentation of Orchid Species in Kedah, Malaysia

Azimah Abd Rahman[1]*, Farah Alia Nordin[2], Muhammad Wafiy Adli Ramli[1] and Nurhafizul Abu Seri[1]

[1]Geoinformatic Unit, Geography Section, School of Humanities, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia
[2]School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia

ABSTRACT

The documentation of orchid species in Kedah, Malaysia, highlights a remarkable biodiversity characterized by 130 species spread across five subfamilies and 61 genera. Significant ecological areas like Gunung Jerai, Ulu Muda Forest Reserve, and Langkawi UNESCO Global Geopark serve as critical habitats, hosting rare and endemic species such as Paphiopedilum exul and Spathoglottis hardingiana. Advanced methodologies, including DNA barcoding and micromorphological analysis, have been utilized to enhance taxonomic precision and elucidate evolutionary relationships among these orchids. Despite these advances, orchid diversity in Kedah faces serious threats from habitat loss due to deforestation, climate change, and illegal poaching. These anthropogenic pressures underline the pressing need for effective conservation strategies. The study identifies limestone hills and montane forests as biodiversity hotspots that require urgent protection. By promoting sustainable practices and addressing habitat degradation, this research underscores the vital importance of integrated conservation strategies to safeguard the unique orchid flora of Kedah for future generations. Furthermore, it advocates for increased awareness, community involvement, and the establishment of a centralized database to facilitate ongoing research and conservation efforts. Ultimately, this documentation serves as a foundational resource for future taxonomic studies, ecological assessments, and biodiversity conservation initiatives, contributing to policy recommendations aimed at preserving and restoring Kedah's rich orchid heritage.

Keywords: Conservation, DNA barcoding, endemic species, habitat loss, limestone hills, orchid diversity

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E-mail addresses:
azimahrahman@usm.my (Azimah Abd Rahman)
farahalianordin@usm.my (Farah Alia Nordin)
mwafiyadli@usm.my (Muhammad Wafiy Adli Ramli)
nurhafizul.abuseri97@gmail.com (Nurhafizul Abu Seri)
* Corresponding author

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INTRODUCTION

Orchids (family Orchidaceae) are among the most diverse and widely distributed plant families, with approximately between 30,000 and 35,000 species globally (Choudhary et al., 2023; Mirioba et al., 2024). In Malaysia, orchid diversity is well-documented, with over 1,000 recorded species in Peninsular Malaysia and up to 3,000 species across Sabah and Sarawak including both terrestrial and epiphytic varieties (Bakar et al., 2023). Their ecological significance, aesthetic value, and economic importance in horticulture have made orchids a subject of extensive research. However, despite numerous studies documenting Malaysia's orchid flora, existing research has primarily focused on broad national-scale assessments or specific biodiversity hotspots like Sabah and Sarawak (Bakar et al., 2023; Besi et al., 2023b). The orchid diversity of Kedah, a state rich in ecological variation, remains understudied and lacks a comprehensive scientific review.

Historically, orchid documentation in Malaysia dates back to colonial botanical expeditions, where species descriptions were scattered across multiple publications and herbarium records (McCormack, 2018). Although subsequent studies have improved species identification and classification, Kedah's orchid diversity has received limited attention, with records often confined to local botanical surveys or unpublished field notes. Research and conservation initiatives in states such as Selangor, Sarawak, and Perlis have led to significant publications on orchid biodiversity, particularly in specific habitats like limestone hills and ecotourism sites, Kedah has not yet received similar attention (Go et al., 2020). The state's diverse landscapes including lowland dipterocarp forests, mangrove swamps, highland forests, and unique limestone formations suggest that Kedah hosts a significant yet underexplored variety of orchid species (Besi et al., 2019; Mirioba et al., 2024).

Moreover, anthropogenic threats such as deforestation, land conversion for agriculture, and illegal orchid collection pose increasing risks to native orchid populations in Kedah (Bakar et al., 2023; Jaafar et al., 2020). Conservation initiatives are hindered by the lack of a centralized database and insufficient documentation of species distribution and status. Without proper records, prioritizing conservation efforts and understanding species vulnerability remains challenging. Thus, this review aims to provide a systematic overview of species diversity, distribution patterns, and conservation challenges of orchids in Kedah. By examining existing records and synthesizing relevant findings, this study seeks to highlight the richness of orchid flora in Kedah, identify knowledge gaps, and assess potential conservation priorities. Given the increasing environmental pressures on orchid habitats, this documentation will serve as a foundation for future taxonomic research, ecological assessments, and biodiversity conservation efforts. Additionally, the insights gained from this review may contribute to policy recommendations, habitat restoration strategies, and sustainable orchid conservation initiatives in Malaysia.

RESULTS AND DISCUSSION

Taxonomic Distribution of Orchid Species

Major research on Malaysian orchids has focused on ecosystems such as limestone forests, peat swamp forests, hill forests, lowland forests, montane forests, coastal heath forests, and logged forests (Besi et al., 2022). Previous study in Gunung Jerai, Kedah, documented 74 orchid species across 40 genera, including 11 new records for Kedah and two new records for Malaysia. Orchid diversity in Gunung Jerai was categorized into four main subfamilies which are Apostasioideae, Cypripedioideae, Epidendroideae, and Orchidoideae. The orchid species found in this area was in the summit region's hill heath forest ecosystem, which is characterized by high humidity and cooler temperatures at elevations above 700 meters. This unique habitat, formed by granite and quartzite geology, supports a diverse range of orchids, with the study identifying 74 species across all five subfamilies of Orchidaceae despite covering only 0.34% of the area. The specific ecological conditions of this isolated mountainous environment are crucial for the growth and reproduction of these orchids, highlighting the need for further research to understand their habitat preferences and conservation requirements (Auyob et al., 2016).

Orchids are an important group in biodiversity conservation due to their inherent characteristics and human influence. They are well-known for their complex life histories, distinctive floral structures, and specialized pollination syndromes (Zhang et al., 2022). However, the classification of orchid species is often complicated by their high genetic variability, hybridization, and diverse ecological adaptations, leading to ongoing taxonomic revisions and new species discoveries. A total of 61 unique genera, including *Apostasia*, *Paphiopedilum*, and *Acheirostylis*, have been documented, indicating substantial biodiversity. The total number of species stands at 130, with classifications for variations and subspecies, underscoring the impressive diversity of orchid flora (Table 1).

According to Table 1 author's found that, the diversity of orchids in Kedah is considerable, including five subfamilies including Apostasioideae, Cypripedioideae, Epidendroideae, Orchidoideae, and Vanilloideae. While map of orchid distribution in Kedah by species count (Figure 1) illustrates the distribution of orchid species in Kedah based on the number of species recorded at each location. In Kedah, orchid species are primarily found in important ecological areas, including as Gunung Jerai (previously Kedah Peak), the Ulu Muda Forest Reserve, and the Langkawi UNESCO Global Geopark. Gunung Jerai, a prominent limestone massif, harbours a unique montane ecosystem characterized by high humidity, well-drained soils, and cooler temperatures, making it an ideal habitat for epiphytic and lithophytic orchids. A study documented 74 orchid species from 40 genera on Gunung Jerai's summit, including 11 new records for Kedah and two new records for Malaysia, indicating the area's rich orchid diversity (Auyob et al., 2016). The Ulu Muda Forest Reserve, an extensive lowland dipterocarp forest, is known for its rich biodiversity, stable microclimate, and high canopy cover, which provide optimal conditions for terrestrial and epiphytic orchid species. The forest supports a variety of wildlife, including large mammals such as elephants and tapirs, and has been identified as a hotspot for orchid diversity, with species like *Bulbophyllum meson* being recorded (Bakar et al., 2023).

Meanwhile, Langkawi's habitat offers a unique environment for orchid species, particularly those adapted to limestone ecosystems, such as *Spathoglottis hardingiana*. Once thought to be restricted to the limestone vegetation of Pulau Langkawi, this species has now been recorded on the mainland, where individuals are significantly larger almost two to three times the size of those found on the island. The limestone formations of Langkawi, part of the Machinchang Formation, share geological similarities with the limestone outcrops of the Baling-Pengkalan Hulu complex in Kedah. Both formations originated over 500 million years ago, creating specialized karst habitats that support diverse orchid species. The floristic similarities between Langkawi, Perlis, and southern Thailand suggest a shared species composition across these regions due to their common geological history. The discovery of *S. hardingiana* in both Langkawi and Baling highlights the adaptability of orchids to ancient limestone substrates, which are characterized by high calcium carbonate content, fluctuating moisture levels, and microclimatic conditions that influence species distribution and growth patterns (Bakar et al., 2023). A comprehensive study by Ong and Nordin (2017) provided an updated description of *S. hardingiana* in Peninsular Malaysia, enhancing understanding of its morphological characteristics and aiding in accurate identification for conservation purposes.

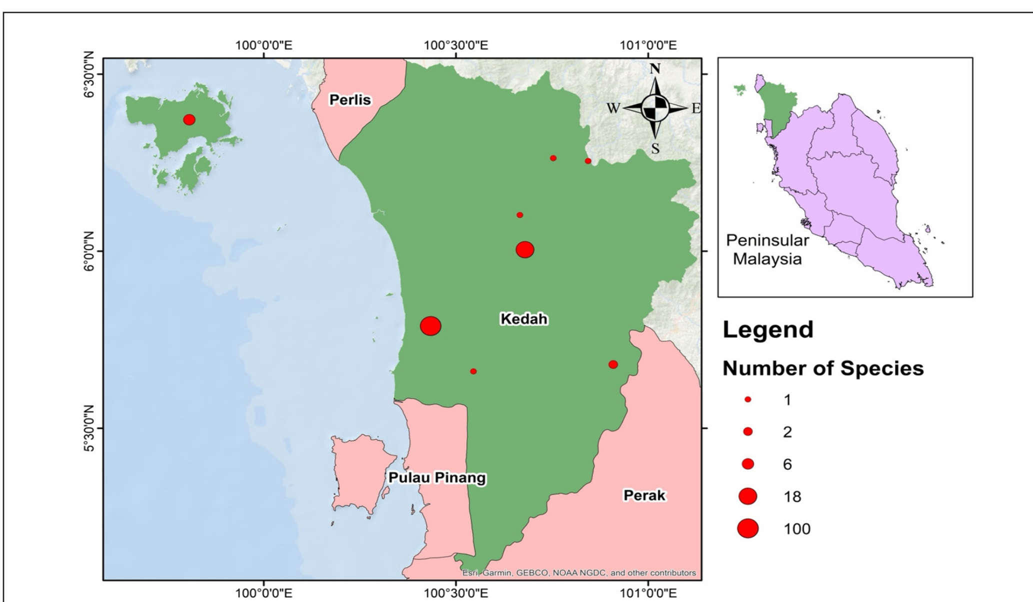

Figure 1. Map of orchid distribution in Kedah by species count

Table 1
Orchids documented in Kedah, Malaysia

Subfamilies	Genera	Species	Location	References
Apostasioideae	*Apostasia*	*Apostasia nuda* R.Br.	Gunung Jerai	1, 4
Cypripedioideae	*Paphiopedilum*	*Paphiopedilum callosum* var. *sublaeve* (Rchb.*f.*) P.J.Cribb *Paphiopedilum exul* *Paphiopedilum niveum* (Rchb.*f.*) Stein*	Bukit Pedu, Gunung Jerai	1, 4, 7, 10
Epidendroideae	*Acheirostylis*	*Acheirostylis goldschmidtiana* Schltr.	Gunung Baling, Gunung Pong,	4
	Acriopsis	*Acriopsis liliifolia* (J.Koenig) Ormerod*	Widespread in Peninsular	4
	Aerides	*Aerides krabiensis* Seidenf. *Aerides odorata* Lour.*	Gunung Baling	4
	Agrostophyllum	*Agrostophyllum majus* Hook.*f.* *Agrostophyllum stipulatum* (Griff.) Schltr. subsp. *Stipulatum** *Agrostophyllum stipulatum* subsp. *bicuspidatum* (J.J.Sm.) Schuit.*	Gunung Jerai	1, 4, 5
	Ania	*Ania penangiana* (Hook.*f.*) Summerh.*	Kedah	4, 5
	Appendicula	*Appendicula anceps* Blume *Appendicula cornuta* Blume *Appendicula reflexa* Blume	Gunung Jerai	1, 3
	Arundina	*Arundina graminifolia* (D.Don) Hochr.	Gunung Jerai	1
	Ascidiiera	*Ascidiiera longifolia* (Hook.*f.*) Seidenf.	Gunung Jerai	1
	Bromheadia	*Bromheadia finlaysoniana* (Lindl.) Miq. *Bromheadia rupestris* Ridl.	Gunung Jerai	1, 3
	Bryobium	*Bryobium hyacinthoides* (Blume) Y.P.Ng & P.J.Cribb	Gunung Jerai	1
	Bulbophyllum	*Bulbophyllum apodum* Hook.*f.* *Bulbophyllum bakhuizenii* Steenis *Bulbophyllum brevipes* Ridl. *Bulbophyllum cheiropetalum* Ridl. *Bulbophyllum cleistogamum* Ridl., J. Linn. Soc.* *Bulbophyllum dayanum* Rchb.*f.* *Bulbophyllum farinulentum* J.J.Sm.	Gunung Jerai; Gunung Fakir Terbang	1, 4, 5

Table 1 *(continue)*

Subfamilies	Genera	Species	Location	References
		Bulbophyllum gibbosum (Blume) Lindl. *Bulbophyllum inunctum* J.J.Sm *Bulbophyllum lilacinum* Ridl. *Bulbophyllum linearifolium* King & Prantl. *Bulbophyllum medusae* (Lindl.) Rchb.*f.** *Bulbophyllum meson* J.J.Verm., Schuit. & de Vogel *Bulbophyllum planibulbe* (Ridl.) Ridl.* *Bulbophyllum purpurascens* Teijsm. & Binn. *Bulbophyllum sigaldiae* Guillaumin *Bulbophyllum uniflorum* (Blume) Hassk.		
	Callostylis	*Callostylis pulchella* (Lindl.) S.C.Chen & Z.H.Tsi *Callostylis rigida* Blume, Bijdr. Fl. Ned.*	Gunung Jerai	1, 5
	Campanulorchis	*Campanulorchis pecilis* (Rchb.*f.* ex Hook.*f.*) Y.P.Ng & P.J.Cribb	Gunung Jerai	1
	Cephalantheropsis	*Cephalantheropsis obcordata* (Lindl.) Ormerod	Gunung Jerai	1
	Ceratostylis	*Ceratostylis subulata* Blume *Cleisostoma sp.*	Gunung Baling, Gunung Jerai,	1, 4
	Claderia	*Claderia viridiflora* Hook.*f.*	Gunung Jerai	1
	Cleisomeria	*Cleisomeria lanata* (Lindl.) Lindl. ex G.Don*	Kedah	4
	Coelogyne	*Coelogyne asperata** *Coelogyne cummingii* Lindl. *Coelogyne foerstermannii* Rchb.*f.* *Coelogyne prasina* Ridl. *Coelogyne rochussenii* de Vriese *Coelogyne swaniana* Rolfe *Coelogyne trinervis* Lindl	Gunung Jerai; Pulau Langkawi	1, 2, 4
	Corymborkis	*Corymborkis veratrifolia* (Reinw.) Blume*	Widespread in Peninsular	4
	Crepidium	*Crepidium calophyllum* (Rchb.*f.*) Szlach*	Kedah	3, 5
	Cylindrolobus	*Cylindrolobus biflorus* (Griff.) Rauschert*	Kedah	5
	Cymbidium	*Cymbidium haematodes* Lindl. *Cymbidium testaceum* (Lindl.) Hook.*	Kedah	2, 4

Table 1 *(continue)*

Subfamilies	Genera	Species	Location	References
	Dendrobium	*Dendrobium aloifolium* (Blume) Rchb.*f.**	Gunung Jerai, Sungai Petani	1, 4, 5, 6
		Dendrobium angustifolium (Blume) Lindl.		
		Dendrobium antennatum		
		Dendrobium crumenatum Sw.		
		Dendrobium derryi Ridl.*		
		Dendrobium hughii Rchb.*f.*		
		Dendrobium indragiriense Schltr.*		
		Dendrobium leonis (Lindl.) Rchb.*f.**		
		Dendrobium linguella Rchb.*f.**		
		Dendrobium lobbii Teijsm. & Binn.*		
		Dendrobium pachyphyllum (Kuntze) Bakh.*f.**		
		Dendrobium pachyglossum C.S.P.Parish & Rchb.*f.* *Dendrobium plicatile* Lindl.*		
		Dendrobium sanguinolentum Lindl.		
	Dendrochilum	*Dendrochilum gracile* (Hook.*f.*) J.J.Sm.	Gunung Jerai	1
		Dendrochilum simile Blume		
	Dienia	*Dienia ophrydis* (J.Koenig) Seidenf.	Gunung Jerai	1
	Dilochia	*Dilochia wallichii* Lindl.	Gunung Jerai	1
	Dipodium	*Dipodium pictum* (Lindl.) Rchb.*f.*	Gunung Jerai	1
	Eria	*Eria diluta* Ridl.	Gunung Jerai	1, 4
		Eria javanica (Sw.) Blume*		
		Eria neglecta Ridl.		
		Eria nutans Lindl.		
		Eria pilifera Ridl.		
	Grosourdya	*Grosourdya appendiculata* (Blume) Rchb.*f.**	Kedah	4
	Ludisia	*Ludisia discolor* (Ker Gawl.) Blume*	Kedah	5
	Liparis	*Liparis elegans* Lindl	Gunung Jerai	1, 3
		Liparis geophila Schltr.		
		Liparis maingayi (Hook.*f.*) Ridl.*		
		Malleola sylvestris (Ridl.) Garay		

Table 1 *(continue)*

Subfamilies	Genera	Species	Location	References
	Luisia	*Luisia brachystachys* (Lindl.) Blume* *Luisia zollingeri Rchb.f.*	Pulau Langkawi	4
	Malleola	*Malleola sylvestris* (Ridl.) Garay	Gunung Jerai	1
	Nephelaphyllum	*Nephelaphyllum tenuiflorum* Blume	Gunung Jerai	1
	Oberonia	*Oberonia sp.**	Kedah	4
	Oxystophyllum	*Oxystophyllum carnosum* Blume*	Kedah	4
	Phaius	*Phaius amboinensis* (Blume)*	Kedah	4
	Pholidota	*Pholidota imbricata* (Hook.)*	Kedah	4
	Pinalia	*Pinalia floribunda* (Lindl.) Kuntze *Pinalia tenuiflora* (Ridl.) J.J.Wood	Gunung Jerai	1, 4
	Plocoglottis	*Plocoglottis lowii* Rchb.*f.*	Gunung Jerai	1
	Podochilus	*Podochilus microphyllus* Lindl. *Podochilus muricatus* (Teijsm. & Binn.) Schltr. *Podochilus tenuis* (Blume) Lindl.	Gunung Jerai	1
	Porpax	*Porpax elwesii* (Rchb.*f.*) Rolfe	Gunung Jerai	1
	Prenanthera	*Prenanthera elongata* (Blume) Lindl.* *Renanthera histrionica* Rchb.*f.**	Kedah	4
	Sarcoglyphis	*Sarcoglyphis comberi* (J.J.Wood) J.J.Wood* *Spathoglottis hardingiana* C.S.P.Parish and Rchb.*f.*	Pulau Langkawi	4
	Spathoglottis	*Spathoglottis affinis* de Vriese *Spathoglottis aurea* Lindl. *Spathoglottis plicata* Blume	Gunung Jerai	1, 8, 11
	Taeniophyllum	*Taeniophyllum pusillum* (Willd.) Seidenf. & Ormerod*	Kedah	4
	Thrixspermum	*Thrixspermum centipeda* Lour.	Gunung Jerai	1
	Trichoglottis	*Trichoglottis lanceolaria* Blume	Gunung Jerai	1
	Trichotosia	*Trichotosia ferox* Blume *Trichotosia gracilis* (Hook.*f.*) Kraenzl. *Trichotosia pauciflora* Blume *Trichotosia poculata* (Ridl.) Kraenzl	Gunung Jerai	1, 4

Table 1 *(continue)*

Subfamilies	Genera	Species	Location	References
	Tropidia	*Tropidia curculigoides* Lindl	Gunung Jerai	1, 4
	Vandopsis	*Vandopsis gigantea* (Lindl.) Pfitzer	Pulau Langkawi	4
Orchidoideae	*Anoectochilus*	*Anoectochilus albolineatus* C.S.P.Parish& Rchb.*f.* *Anoectochilus geniculatus* Ridl. *Anoectochilus sanguineus* P.T.Ong & P.O'Byrne*	Gunung Jerai	1, 4
	Corybas	*Corybas geminigibbus* J.J.Sm. *Corybas calopeplos* Dransfield & Smith*	Gunung Jerai	1, 9
	Cryptostylis	*Cryptostylis arachnites* (Blume) Hassk.	Gunung Jerai	1
	Goodyera	*Goodyera viridiflora* (Blume) Blume	Gunung Jerai	1, 3
	Habenaria	*Habenaria rhodocheila* Hance*	Kedah	3
	Peristylus	*Peristylus monticola* (Ridl.) Seidenf.*	Kedah	3
	Zeuxine	*Zeuxine affinis* (Lindl.) Benth. ex Hook.*f.* *Zeuxine gracilis* (Breda) Blume	Gunung Jerai	1, 3
Vanilloideae	*Lecanorchis*	*Lecanorchis malaccensis* Ridl.	Gunung Jerai	1

Note. 1- Auyob et al. (2016); 2- Yoh et al. (2022); 3- Nordin et al. (2021); 4- Bakar et al. (2023); 5- Besi et al. (2019); 6- Rahim and Mohd (2021); 7- Besi et al. (2022); 8- Nordin et al. (2022); 9- Go et al. (2015); 10- Besi et al. (2021); 11- Ginibun et al. (2018a). Symbols (*) indicate locations not specifically mentioned

Rare and Endemic Species

Besi et al. (2019) documented several notable orchid species in Kedah, highlighting the region's rich biodiversity. *Agrostophyllum stipulatum* (Griff.) Schltr. subsp. *stipulatum* and *Agrostophyllum glumaceum* Hook.*f.* are commonly found in lowland and montane forests, typically growing epiphytically on fallen trees. The subspecies *Agrostophyllum stipulatum* subsp. *bicuspidatum* (J.J.Sm.) Schuit. shares similar habitats and growth patterns. *Ania penangiana* (Hook.*f.*) Summerh., in contrast, is less common and thrives in grassy areas of hills and montane forests on dune-originated soils. *Trichoglottis lanceolaria* Blume and *Trichotosia gracilis* (Hook.*f.*) Kraenzl. are also frequently encountered in hill and montane forests, with epiphytic growth on fallen trees. The diversity of orchid species in Kedah is exemplified by *Spathoglottis hardingiana*, a species found in prominent locations such as Gunung Baling and Pulau Timun in Langkawi. Nordin et al. (2022) emphasize the ecological significance and unique floral characteristics of this species, along with others in the Dwarf *Spathoglottis* group. Through phylogenetic analysis, their research not only reinforces the classification of these orchids but also sheds light on their evolutionary relationships, offering valuable insights into the biodiversity of orchids in Kedah.

Nordin et al. (2021) conducted a study focusing on the orchid flora of Gunung Ledang, documenting a total of 122 species, including five endemic to Peninsular Malaysia and two hyper-endemic species unique to the region, thereby underscoring the mountain's exceptional biodiversity. Notably, 26 species (67%) corresponded with records by Ridley (1901), and 65 species (83%) aligned with Turner's (1995) checklist, illustrating both the persistence of certain species and shifts in their distribution over time. The study employed advanced methodologies, such as field and stereo microscopy, to enhance species identification while minimizing the collection of living specimens, demonstrating adherence to contemporary conservation practices. Furthermore, the identification of 30 new records for the locality highlights the ongoing potential for species discovery in under-explored regions. These findings not only enrich the understanding of orchid diversity in Gunung Ledang but also underscore the critical importance of conservation efforts in safeguarding its unique floristic heritage.

Research on the limestone hills of Kedah showed a great diversity of orchids, with 56 species from 37 genera identified in five important locations: Gunung Fakir Terbang, Gunung Batu Putih, Gunung Pong, Gunung Baling, and Gunung Pulai. Twelve of these species were newly discovered in Kedah, while *Bulbophyllum meson* and *Luisia brachystachys* were discovered in Malaysia for the first time. Peninsular Malaysia is home to three species that have been identified as endemic: *Anoectochilus sanguineus*, *Cheirostylis goldschmidtiana*, and *Phalaenopsis appendiculata*. These environments are important ecologically, as evidenced by the rediscovery of *Cheirostylis goldschmidtiana* and the endangered *Paphiopedilum niveum*. Gunung Batu Putih, Gunung Baling, and

Gunung Fakir Terbang were found to be the most diversified sites (Bakar et al., 2023). The study's use of heat maps to identify geographical hotspots of orchid diversity, particularly at Gunung Fakir Terbang, provides valuable insights into species distribution that can guide future conservation efforts.

Besi et al. (2022) identify Bukit Pedu in Kedah as a critical habitat for orchid diversity, particularly highlighting the rare species *Paphiopedilum exul*, which thrives in the shaded, dry conditions of limestone hill forests. This finding underscores the ecological significance of Bukit Pedu in contributing to the rich biodiversity of orchids in northern Peninsular Malaysia. The presence of *P. exul* not only enriches the documented orchid flora of the region but also emphasizes the urgent need for conservation efforts in light of threats such as deforestation and land development. These findings reinforce the importance of safeguarding vulnerable habitats to ensure the preservation of unique orchid species. *P. exul* is a rare and endangered orchid species that requires targeted conservation efforts due to habitat loss and overcollection. Research by Imsomboon and Thammasiri (2017) examined the effects of pH and sucrose on the seed germination of *P. exul*, identifying optimal conditions that could enhance propagation efforts. Their findings are crucial for ex-situ conservation, particularly for seedling production in controlled environments. Furthering these efforts, Imsomboon and Thammasiri (2020) investigated cryopreservation techniques for *P. exul* seeds, providing strategies for long-term preservation. This method ensures genetic material is retained for future restoration initiatives, making cryopreservation a vital tool in safeguarding the species from extinction.

Corybas geminigibbus and *Corybas calopeplos*, two rare orchids of mossy montane forests, play significant ecological roles and require targeted conservation measures. These species are highly specialized, thriving in thick humus layers within montane ecosystems, which are characterized by cool temperatures, high humidity, and limited soil disturbance (Go et al., 2015). Their restricted distribution *C. geminigibbus* being confined to a single locality in Kedah and *C. calopeplos* found only in two locations in Kedah and Pahang highlights their vulnerability to environmental changes and habitat degradation. As part of the montane orchid flora, these species contribute to the stability of their ecosystems by forming intricate relationships with fungal symbionts necessary for seed germination and nutrient uptake (Swarts & Dixon, 2009). Additionally, their presence serves as an indicator of ecosystem health, as montane orchids are highly sensitive to climate fluctuations and habitat disturbances (Dixon et al., 2003). Given their limited range and specialized habitat requirements, urgent conservation efforts are needed to prevent further population declines. Protecting montane forest habitats from deforestation, land conversion, and climate change impacts is critical. Ex-situ conservation strategies, such as in-vitro propagation and mycorrhizal symbiont research, could enhance their survival prospects and facilitate potential reintroduction efforts (Rasmussen et al., 2015). Furthermore, integrating these species into conservation policies and raising awareness about their ecological importance among local communities can contribute to long-term protection initiatives. Without immediate conservation action, the survival of *C. geminigibbus* and *C. calopeplos* remains at risk, threatening the broader biodiversity of Malaysia's montane ecosystems.

Advanced Methodologies

Yoh et al. (2022) conducted an in-depth study on *Coelogyne* species in Peninsular Malaysia, yielding significant findings that advance understanding of this genus. The study identified two primary clades based on morphological and molecular analyses: the first comprising the sections *Longifoliae*, *Speciosae*, and *Fuliginosae*, and the second encompassing *Flaccidae*, *Coelogynae*, *Tomentosae*, and *Verrucosae*. These results highlight distinct evolutionary relationships among the groups. A morphological similarity of 61.9% among the examined species underscores the complexities in species delimitation due to substantial overlap in traits. Furthermore, the findings corroborate the taxonomic classifications proposed by Seidenfaden and Wood (1992), reinforcing their validity. The study underscores the necessity for continued morphological and molecular research to address unresolved taxonomic ambiguities and improve species delimitation within the genus.

DNA barcoding has significantly improved orchid taxonomy by enabling precise species identification, particularly for morphologically similar taxa. DNA barcoding has been widely recognised as an effective tool for the accurate identification of medicinal orchids, particularly when morphological features are indistinguishable in processed plant materials. Studies have demonstrated that genetic markers such as ITS, matK, rbcL, and trnH-psbA are reliable for distinguishing closely related orchid species, aiding in conservation and trade regulation. The establishment of a barcode library containing over 7,000 sequences has significantly improved the authentication of medicinal orchids in Asia (Raskoti & Ale, 2021). This technique has been instrumental in resolving taxonomic ambiguities, such as distinguishing cryptic species within genera like *Dendrobium* and *Bulbophyllum* (Xu et al., 2015).

Molecular DNA barcoding, combined with botanical taxonomy, serves as a crucial tool for the identification and conservation of orchids. This approach is particularly valuable in distinguishing species that are morphologically similar, supporting both biodiversity assessments and conservation efforts. A modified CTAB protocol is commonly used for DNA extraction, followed by the amplification of key barcoding regions such as ITS, matK, rbcL, and trnH-psbA, which enhance species classification accuracy. DNA sequencing data are typically analysed using bioinformatics tools, such as BioEdit software for sequence assembly and the BLAST algorithm on the NCBI database for species identification. Studies have demonstrated that molecular barcoding data align closely with traditional taxonomic classifications, reinforcing its reliability as a species identification method. Additionally, research suggests that the combination of ITS and matK provides greater precision in differentiating orchid species compared to other genetic markers. Beyond identification, DNA barcoding contributes to conservation efforts by enabling the creation of species reference databases, which can be used to track genetic diversity and monitor species distribution (Tsaballa et al., 2023). As habitat loss and illegal trade continue to threaten global orchid diversity, the integration of DNA barcoding with conservation strategies is essential for long-term species protection and ecological sustainability.

According to Karbarz et al. (2024), DNA barcoding is an effective tool for determining the genetic identity of endangered *Paphiopedilum* orchids. Their study evaluated five loci (matK, rbcL, ITS2, atpF-atpH, and trnH-psbA) as potential molecular markers. Among single-locus barcodes, matK was the most effective, correctly identifying 64% of species. However, matK + ITS2, matK + rbcL, and matK + trnH-psbA were recommended as complementary tools alongside morphological data for species identification. Given the threats of overexploitation and illegal trade, precise genetic identification is essential for conservation. The findings emphasize the need for broader testing and barcode selection based on species representation within the genus. According to Lal et al. (2025), DNA barcoding is an effective technique for molecular-level identification in plants, particularly orchids, which exhibit high genetic variability despite morphological similarities. Their study assessed the effectiveness of universal chloroplast gene markers (matK and rbcL) for identifying *Aerides multiflora* and *Rhynchostylis retusa* at the species level. Phylogenetic analysis revealed 99.4% sequence alignment for matK in A. multiflora and 99.87% alignment for matK in *R. retusa*, while the rbcL region showed 100% identity with *R. retusa*, *C. peduncularis*, and *A. praemorsa*. These findings confirm the reliability of matK and rbcL as DNA barcodes for authenticating these orchid species.

DNA barcoding is a powerful tool for species identification and phylogenetic studies, particularly in plants with closely related species that exhibit high morphological similarity. While universal barcode markers such as rbcL and matK are widely used, their effectiveness in distinguishing species within the same genus can be limited. Recent research has shown that alternative genetic loci, such as nuclear ribosomal DNA (nrDNA) markers, may provide higher resolution for phylogenetic classification. By analysing multiple genetic regions, scientists can improve species identification, address phylogenetic incongruences, and enhance the accuracy of evolutionary studies in plant biology (Chattopadhyay et al., 2017). Another importance of DNA barcoding is in identifying plant species with high morphological similarity, such as *Cymbidium* orchids, which have significant ornamental and commercial value. Research comparing multiple genetic loci, including plastid (matK, rbcL, psbA-trnH, atpF-atpH) and nuclear (ITS) markers, has shown that ITS provides the highest resolution for distinguishing *Cymbidium* species. This marker exhibits greater genetic divergence, a well-defined barcoding gap, and a high success rate in species identification. Additionally, ITS-based phylogenetic analysis aligns with traditional morphological classification, making it a reliable tool for both species authentication and evolutionary studies in *Cymbidium* orchids (Chen et al., 2024).

Additionally, Rajaram et al. (2019) demonstrated the effectiveness of DNA barcoding in identifying endangered *Paphiopedilum* species from Peninsular Malaysia. Their study evaluated four DNA markers (rbcL, matK, ITS, and trnH-psbA) and found matK to be the most reliable, exhibiting 100% sequence quality, BLASTn accuracy, and species resolution in phylogenetic analysis. While ITS and trnH-psbA showed moderate effectiveness, rbcL had limited variation. The study suggests that a combination of these markers can aid in preliminary species identification, with matK offering the highest accuracy for definitive classification. This highlights the value of DNA barcoding in conservation and taxonomy of *Paphiopedilum* orchids. Ginibun et al. (2018b) explored the use of DNA barcoding for species identification in *Spathoglottis*, a terrestrial orchid with landscaping and commercial value. Due to the morphological diversity of orchids, traditional identification methods are often insufficient. The study examined four chloroplast coding regions (matK, rbcL-a, rpoB, and rpoC1) to assess genetic variation and identified eight haplotype groups for DNA barcoding. A combination of rbcL-a, rpoB, and rpoC1 provided the highest resolution for distinguishing species. Phylogenetic analysis revealed that five Malaysian native species formed a similar group, with *S. plicata* being distinct from *S. microchilina*, *S. affinis*, *S. aurea*, and *S. gracilis*. These findings highlight the usefulness of chloroplast DNA markers in the phylogenetic classification of *Spathoglottis*.

In different research, detailed analyses of floral structures, such as those in *Paphiopedilum barbatum* and *P. callosum* var. *sublaeve*, play a crucial role in distinguishing closely related orchid species, particularly in regions like Kedah. The integration of DNA barcoding with morphological studies has proven effective in improving taxonomic clarity and addressing conservation challenges for endemic or endangered orchids. Employing advanced methodologies, including scanning electron microscopy, not only facilitates micromorphological examination but also aids in understanding ecological distributions. These insights are essential for developing targeted conservation strategies to combat the significant threats posed by habitat loss to orchid diversity in Kedah (Besi et al., 2021). Meanwhile research by Ginibun et al. (2018a) reveals that 78.5% of genetic variation exists within populations, highlighting the critical need to preserve genetic diversity for the survival of orchid species, particularly in Kedah, where similar environmental threats may endanger local populations. The study also underscores the detrimental effects of habitat loss driven by industrialization, a pressing issue faced by native orchids in the region. Findings on the distinct genetic structures among *Spathoglottis plicata* populations suggest that geographical factors play a significant role in genetic differentiation, a principle applicable to other orchid species in Kedah. Moreover, the application of Amplified Fragment Length

Polymorphism (AFLP) markers in this research offers a robust methodological framework for evaluating orchid diversity and conservation status.

Gostel and Kress (2022) highlight the pivotal role of DNA barcoding in species identification, ecological research, evolutionary studies, and conservation. By providing a rapid and accurate method for distinguishing species, especially those with high morphological similarity, DNA barcoding has revolutionized taxonomic classification. Its application extends to understanding species interactions, phylogenetic relationships, and biodiversity patterns, thereby contributing to ecological and evolutionary research. Furthermore, DNA barcoding aids in conservation efforts by facilitating the monitoring of endangered species, preventing illegal wildlife trade, and informing targeted conservation strategies. Advances in sequencing technology and computational tools have enabled large-scale, high-throughput barcoding applications, enhancing species classification and genetic data analysis. The continuous expansion of global DNA barcode databases further strengthens taxonomic frameworks, supports the discovery of new species, and refines existing classifications. As biodiversity faces increasing threats from habitat loss and environmental changes, DNA barcoding remains an indispensable tool for mitigating species extinction and preserving genetic diversity. While Mahadani et al. (2022) mentioned that traditional methods of species identification can be challenging due to morphological similarities, making molecular approaches such as DNA barcoding a reliable alternative. By utilizing genetic markers like ITS, matK, rbcL, and trnH-psbA, researchers can accurately distinguish between closely related species. Among these loci, ITS has been found to be the most efficient for species identification, although some species may require additional methods for precise differentiation. The integration of DNA barcoding with similarity-based and character-based approaches enhances species authentication, contributing to biodiversity conservation, sustainable resource management, and the protection of medicinal plant integrity. As more sequences are added to global databases, DNA barcoding continues to strengthen its role in botanical research, facilitating accurate species classification and supporting efforts in ecological and pharmaceutical studies.

Similarly, micromorphological analysis, particularly through scanning electron microscopy (SEM), has also provided deeper insights into orchid taxonomy. A study examining the labellum surfaces of 21 *Dendrobium* species identified distinct structural features that aid in species differentiation, refining classifications that were once solely based on floral morphology (Burzacka-Hinz et al., 2022). Additionally, seed micromorphology studies have clarified evolutionary relationships within the Orchidaceae family. For example, analysis of seed coat structures in Habenaria and related taxa has offered valuable data for delimiting species boundaries, supporting DNA-based phylogenies (Rewicz et al., 2022). By integrating DNA barcoding and micromorphological analyses, researchers have corrected historical taxonomic errors and uncovered new species, reinforcing the importance of combining molecular and morphological approaches for comprehensive orchid systematics.

Micromorphological analysis using light and SEM has revealed key epidermal structures that aid in orchid species identification and phylogenetic classification. Variations in labellum surface striations, secretory trichomes, and papillae contribute to distinguishing taxa. The presence of secretory cells and calcium oxalate crystals within the labellum suggests adaptations related to pollination strategies. For instance, in *Ophrys*, dense labellum hairs mimic insect morphology, potentially enhancing pollinator attraction. The invaginations of the labellum forming the spur, along with specific epidermal features, highlight morphological diversity across genera. Cluster analysis (UPGMA) has further refined species classification by evaluating micromorphological traits, reinforcing their significance in resolving taxonomic ambiguities and understanding evolutionary relationships (Şeker et al., 2016). While study by Aytar et al. (2024) highlights the significance of morphometric and FTIR chemical analyses in distinguishing orchid species and understanding their seed characteristics. Variations in seed size, embryo size, and cavity percentage contribute to taxonomic differentiation, with species of the same genus clustering together. FTIR spectral analysis confirms the familial classification of the examined species while revealing interspecific variations in absorbance values, particularly between Neotinea tridentata and other orchids. Principal Component Analysis (PCA) based on FTIR data further supports these findings, demonstrating its potential in refining taxonomic classification and enhancing conservation strategies through improved seed propagation techniques.

Threats to Orchid Species

Orchid populations are declining worldwide due to habitat loss and climate change, with conservation efforts hindered by limited knowledge of their ecological requirements and unresolved taxonomic issues (Kindlmann et al., 2023). Beyond their taxonomic significance, orchids are particularly vulnerable to habitat destruction, climate change, and unsustainable harvesting, which is often illegal and undocumented (Fay, 2018; Gale et al., 2018). Their intricate biological relationships with pollinators, mycorrhizal fungi, and specific habitat conditions make them highly sensitive to environmental disturbances. Conservation efforts are therefore critical not only for preserving orchid diversity but also for maintaining their ecological functions within natural ecosystems. Initiatives aimed at protecting orchids can help mitigate the impacts of habitat loss, safeguard rare species, and promote sustainable management practices to ensure their survival for future generations. While Bakar et al. (2023) stated that Kedah's limestone hills, which harbour rare and endemic orchid species, face significant threats from anthropogenic activities. Habitat destruction due to quarrying, deforestation, and land-use changes poses a severe risk to orchid populations, potentially leading to species extinction. Quarrying operations, in particular, result in irreversible damage by altering microhabitats, reducing suitable growing conditions, and disrupting ecological interactions essential for orchid survival. Additionally, deforestation driven by agricultural expansion and infrastructure development further fragments orchid habitats, making species more vulnerable to environmental stressors. The lack of comprehensive documentation on orchid diversity in these areas exacerbates the conservation challenge, as unrecorded species may be lost before their ecological significance is fully understood. Urgent conservation measures are needed to mitigate these threats and protect the fragile orchid ecosystems of Kedah's limestone hills.

The survival of wild orchids in Malaysia is increasingly threatened by habitat destruction, illegal poaching, and climate change. Rapid deforestation and land development, particularly through logging and construction, have led to significant habitat loss. Terrestrial orchids are especially vulnerable, as they are often uprooted or buried during land conversion. A striking example is *Gastrodia tembatensis*, which lost its original habitat in Terengganu due to dam construction, emphasizing how large-scale infrastructure projects can endanger rare species. This condition could also happen to orchids in Kedah. Illegal collection of orchids also poses a severe threat, with rare and visually striking species such as Slipper Orchids being heavily targeted for private collections and commercial trade. The rise of social media has worsened this issue, providing an unregulated marketplace where poachers can easily sell wild orchids, making enforcement efforts difficult. Additionally, climate change further threatens orchid populations by disrupting ecological conditions necessary for their survival. Rising temperatures, decreasing humidity, and prolonged dry spells have negatively impacted moss-rich montane environments, which serve as critical habitats for many orchid species. Without urgent conservation measures, the continued loss of these delicate ecosystems may push several wild orchids toward extinction before they can be adequately studied or preserved (Chacko, 2025).

However, non-native orchid species, such as *Dendrobium antennatum*, while not native to Malaysia, have also been observed in certain regions, such as Kedah. This species, native to Australia and the Pacific Islands, has occasionally been introduced to Malaysia, often through horticultural activities. Although *D. antennatum* does not naturally occur in Malaysian ecosystems, its presence offers valuable insight into the broader landscape of orchid distribution, particularly in the context of species introduction, hybridization, and conservation. Understanding the distribution of both native and non-native species can shed light on potential conservation challenges, such as the competition with native species and disruption of local habitats. Rahim and Mohd (2021) offer valuable insights into *D. antennatum* does, a notable species within the Orchidaceae family, underscoring its ecological and economic significance in Malaysia. Their study highlights the ornamental value of *D. antennatum*, which is widely cultivated for its aesthetic appeal, thereby contributing to the region's horticultural economy.

However, the identification of *Fusarium sacchari* as a pathogenic threat highlights the challenges faced by cultivators in managing fungal infections, which can significantly impact local growers. Conducted in Sungai Petani and Penang, the study's geographical context underscores the broader implications of disease transmission within adjacent regions, including Kedah. As a foundational investigation into orchid diseases, it highlights the critical need for continued research to monitor and mitigate emerging threats, ensuring the sustainable cultivation and conservation of this valuable species (Rahim & Mohd, 2021). Wild orchids face significant threats due to habitat destruction, environmental changes, and human activities (Bakar et al., 2023). One of the primary concerns is deforestation, which leads to the permanent loss or degradation of habitats where orchids thrive. Logging, quarrying, and land clearing disrupt the delicate ecological interactions that orchids rely on, such as their symbiotic relationships with mycorrhizal fungi, host trees, and pollinators. These disturbances alter climatic conditions, affecting factors like light availability, humidity, and wind patterns, all of which are crucial for orchid survival. Quarrying activities further exacerbate habitat loss by removing vegetation and modifying the landscape, making it difficult for orchid populations to recover. Given the increasing threats to wild orchids, urgent conservation measures are necessary to protect these species. Strict regulations should be implemented to prevent further habitat destruction, while conservation programs must focus on habitat restoration and sustainable land management. Preserving wild orchids is not only essential for maintaining biodiversity but also for safeguarding their ecological and cultural significance for future generations.

In Peninsular Malaysia, undisturbed forests had a significantly higher orchid density (2.433 plants/km^2) than disturbed forests (0.228 plants/km^2) and disturbed forests had a higher diversity (H = 4.934), which was explained by the abundance of epiphytic orchids that colonised fallen trees. Key elements favouring orchid growth and high density were found to be the microclimatic conditions of undisturbed forests, which included temperatures between 27.8 and 31.2°C, humidity levels between 77.1% and 89.6%, and light intensities between 23.8 and 171.7 μmol m−2s−1. However, habitat deterioration resulted from disturbances like logging, with canopy openings causing drastic variations in temperature, humidity, and light intensity (Besi et al., 2023b). While the primary threats to orchid species in Malaysia encompass habitat destruction caused by logging, agricultural land clearance, and infrastructure development, all of which profoundly disrupt their natural environments. Illegal collection and poaching of high-value species such as *Paphiopedilum* and *Phalaenopsis* further intensify their decline, driven by a lucrative market often facilitated through e-commerce platforms. Moreover, climate change poses additional risks to the fragile ecosystems that sustain orchids, while insufficient awareness among local communities about sustainable practices hampers conservation efforts. These combined factors have resulted in the classification of numerous orchid species as threatened or endangered, underscoring the pressing need for effective conservation strategies (Go et al., 2020).

The survival of *Corybas* species in Peninsular Malaysia is increasingly threatened by environmental changes, particularly climate-related factors. The decline of montane moss carpets, which serve as critical microhabitats for these orchids, poses a significant risk to their persistence. Observations indicate that mosses in montane forests are becoming thinner and more fragmented due to rising temperatures, decreasing relative humidity, and prolonged dry spells. Such climatic shifts create unfavourable conditions for *Corybas* species, many of which are narrow endemics with extremely limited distributions. The reduction in suitable habitat not only decreases population sizes but also heightens the vulnerability of these orchids to extinction, as they are unable to adapt quickly to rapidly changing environmental conditions (Go et al., 2015).

Invasive plant species can significantly impact native orchids by competing for essential resources and disrupting critical symbiotic relationships. For instance, invasive plants often release allelopathic compounds that can inhibit the growth of arbuscular mycorrhizal fungi (AMF), which are vital for orchid seed germination and nutrient uptake. Research indicates that allelopathic substances from invasive species can decrease AMF infection rates in native plants, thereby hindering orchid establishment and survival (Guo et al., 2023). Additionally, invasive plants like *Alliaria petiolata* (garlic mustard) produce secondary chemicals toxic to soil microorganisms, including mycorrhizal fungi. This toxicity can lead to physiological stress in native forest understory plants, such as orchids, reducing their population growth rates (Roche et al., 2021). Moreover, the presence of invasive orchids, such as Spathoglottis plicata, has been shown to negatively affect native orchid species through mechanisms like apparent competition. The invasive orchid attracts shared herbivores, which can increase damage to native orchids and contribute to their decline (Recart et al., 2013). These findings underscore the importance of managing invasive species to protect native orchid populations and maintain ecosystem health.

Recommendations for Policy and Conservation Actions

Deforestation in Kedah, driven by logging, agricultural expansion, and urban development, has led to significant habitat loss, particularly in ecologically sensitive areas. Over the past two decades, approximately 9% of forest cover has been lost, equating to more than 33,000 hectares (Jaafar et al., 2020). This loss is especially concerning in critical habitats such as limestone hills and montane forests, which are home to rare and endemic orchids like *Corybas geminigibbus* and *Corybas calopeplos*. The removal of trees not only directly eliminates orchid habitats but also disrupts essential ecological interactions, such as mycorrhizal associations crucial for seed germination and growth (Go et al., 2015).

Furthermore, deforestation alters microclimatic conditions, including humidity, temperature, and soil composition, which are vital for orchid survival. The loss of canopy cover can lead to increased exposure to sunlight and reduced moisture retention, negatively affecting orchids that thrive in shaded, humid environments (Li et al., 2022). Additionally, habitat fragmentation may limit pollinator availability, reducing reproductive success and genetic diversity among orchid populations (Sáyago et al., 2018). Given these threats, urgent conservation measures, such as habitat restoration and the establishment of protected areas, are necessary to safeguard Kedah's orchid diversity.

Climate change poses a significant threat to the orchids of Kedah. Over the past five decades, Malaysia has experienced surface temperature increases ranging from 0.32°C to 1.5°C (Hassan et al., 2023). Additionally, annual rainfall depth in the region is projected to rise by approximately 0.9% per year (Tukimat & Harun, 2015). These climatic shifts can alter microhabitats essential for orchid development. For instance, prolonged dry spells and reduced humidity have been observed to negatively impact moss-rich montane environments, which are crucial for many orchid species (Go, 2015). If current climate trends persist, areas that presently support high orchid diversity may become less suitable, potentially leading to the loss of rare species. The illegal collection of orchids, particularly rare and visually striking species such as *Spathoglottis hardingiana*, poses a significant threat to their populations. Studies have documented the rise of unregulated markets for wild orchids facilitated by social media platforms, where collectors and suppliers engage in the trade of these species (Hinsley et al., 2016). This trend not only threatens species survival but also undermines conservation efforts due to a lack of accountability and enforcement.

Besi et al. (2023a) provided an overview of the key legislations that regulate plant diversity conservation in Malaysia, highlighting the role of various forestry policies and legal frameworks in ensuring sustainable forest management and species protection. The National Forestry Act 1984 (amended 1993) and the National Forestry Policy 1978 serve as the foundation for regulating forest resources, with the primary objective of balancing conservation efforts with timber production. These legislations are reinforced by the Wood-Based Industries Act 1984, which ensures the sustainable development of industries reliant on forest resources. To safeguard biodiversity, the Permanent Reserved Forests (PRF) were established under the National Forestry Act 1984, categorized into 12 functional classes such as Timber Production Forest, Wildlife Sanctuary, and Water Catchment Forest. The management of these areas is under the jurisdiction of the Forest Departments of Peninsular Malaysia (FDPM), whereas national parks and wildlife sanctuaries fall under the authority of the Department of Wildlife and National Parks Peninsular Malaysia (PERHILITAN). In Sabah and Sarawak, conservation laws are dictated by state-specific policies. Sabah enforces the State Forest Policy of 1954 and the Forest Enactment Policy of 1968, while Sarawak adheres to the Statement of Forest Policy of 1954 and the Forests Ordinance of 1954. Additionally, Sarawak's Wildlife Protection Ordinance 1998 plays a crucial role in safeguarding all orchid species, emphasizing their ecological and conservation significance. These legal frameworks collectively demonstrate Malaysia's commitment to protecting its diverse plant species while addressing the challenges posed by habitat loss and industrial expansion.

Strengthening legal frameworks is crucial for safeguarding orchid species and their habitats. Enforcing stricter penalties for illegal collection and designating protected areas for critical habitats can significantly enhance conservation outcomes. Research indicates that while environmental protections exist, they may be ineffective without targeted actions against specific threats to orchids (Scramoncin et al., 2024). Additionally, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) serves as a primary legislative body for orchid conservation worldwide, encompassing all orchid species and representing more than 90% of the plant species protected by the Convention (Li et al., 2024). By implementing and enforcing such legal protections are vital steps in preserving orchid populations and their ecosystems.

Integrating Environmental Impact Assessments (EIAs) into developmental projects within sensitive areas is crucial for the conservation of orchid habitats. EIAs serve as a preventive measure, identifying potential threats to these ecosystems before project initiation, thereby facilitating a balance between development and environmental preservation. Research has demonstrated that habitat fragmentation and anthropogenic disturbances significantly impact orchid diversity and distribution. For instance, a study analysing orchid communities in Australia's Mount Lofty Ranges found that habitat configuration and the presence of exotic species explained a substantial variance in orchid richness and diversity (Martín-Forés et al., 2022). By requiring EIAs, such potential threats can be identified and mitigated proactively, ensuring that development projects do not compromise the integrity of orchid ecosystems.

Implementing incentive programs that reward landowners and local communities for conserving natural habitats can effectively promote sustainable practices. Financial mechanisms, such as Payments for Ecosystem Services (PES), provide compensation to those who manage and protect ecosystems, ensuring the continued provision of vital services. PES schemes have been successfully applied in various contexts, offering economic incentives to landowners for maintaining biodiversity and ecosystem health. For instance, a study highlighted the role of PES in promoting conservation by compensating land users for preserving natural environments, thereby ensuring the flow of ecosystem services (Herbert et al., 2010). In the context of orchid conservation, PES can motivate stakeholders to protect orchid-rich ecosystems. Orchids provide a range of ecosystem services, including cultural, provisioning, supporting, and regulating services. A global analysis of orchid-related ecosystem services found that cultural services were the most documented, followed by provisioning, supporting, and regulating services (Hernández-Mejía, Rosa-Manzano et al., 2024). By recognizing and financially valuing these services, PES programs can encourage the preservation of habitats critical to orchid survival. Additionally, understanding local perceptions and drivers of engagement in PES programs is essential for their success. Research indicates that aligning PES initiatives with community values and providing clear economic benefits can enhance participation and effectiveness (Izquierdo-Tort et al., 2024). Therefore, designing PES schemes that are context-specific and collaboratively developed with local stakeholders can lead to more sustainable conservation outcomes.

In addition, promoting ecotourism that focuses on orchid diversity can generate economic benefits while fostering an appreciation for local biodiversity. Examples include guided tours through orchid habitats, educational sessions on the role of orchids in ecosystems, and participatory citizen science projects. A study in Central Veracruz, Mexico, highlighted that native orchid tourism serves as a tool for conservation, as tourists show interest in learning about orchids and their natural environments, thereby supporting local economies and conservation efforts (Hernández-Mejía, Baltazar-Bernal et al., 2024). Establishing a centralized database is crucial for tracking the distribution, population health, and habitat conditions of orchid species, thereby facilitating effective conservation planning. Such databases provide a foundation for data-driven decision-making, enabling stakeholders to identify population trends and habitat changes over time. For instance, OrchidBase 4.0 compiles whole-genome sequences and annotations of multiple orchid species, serving as a valuable resource for researchers and conservationists (Hsiao et al., 2021). Furthermore, integrating geospatial technologies, such as remote sensing and GIS mapping, can further enhance real-time monitoring and improve conservation strategies. A study demonstrated the use of remote sensing and image processing techniques for identifying and mapping wild orchids, highlighting the potential of these technologies in conservation efforts (Ahmed et al., 2024).

CONCLUSION

The comprehensive documentation of orchid species in Kedah underscores the region's ecological significance as a vital centre of orchid diversity. The identification of unique habitats and the discovery of rare and endemic species highlight Kedah's critical role within Malaysia's broader biodiversity landscape. While advancements in research methodologies have improved species classification and distribution insights, they also reveal pressing threats such as habitat destruction and climate change. Effective conservation measures such as establishing protected areas, engaging local communities in habitat restoration, and implementing targeted policies are crucial to address these challenges. Future research should focus on understudied habitats, the effects of climate change on orchid populations, and the role of community engagement in conservation efforts. By fostering sustainable practices and promoting awareness about the importance of orchid biodiversity, we can ensure the protection and survival of Kedah's unique orchid populations. Ultimately, safeguarding this botanical legacy is essential for maintaining ecological balance and contributing to global biodiversity, serving as a testament to Kedah's rich natural heritage.

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Retrospective Analysis of Equine Traumatic Injury: Patterns and Insights

Zulfitri Naim Abdul Rahim[1], Noraniza Mohd Adzahan[1]*, Intan Shameha Abdul Razak[2], Zunita Zakaria[3] and Puteri Rose Camelia Roselan[1]

[1]*Department of Farm and Exotic Animal Medicine and Surgery, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[2]*Department of Veterinary Pre – Clinical Science, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[3]*Department of Veterinary Pathology & Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

Traumatic injury is a catastrophic and second most frequent emergency condition that is significantly labor intensive and expensive to treat, raising a welfare concern in the equine industry. Even though the incidence is high, there is still a lack of data addressing the prevalence of these traumatic injuries, particularly in Malaysia. Therefore, this study aims to describe the occurrence and patterns of the traumatic injuries experienced by the subpopulation of horses in Malaysia. The records of horses reported to the University Veterinary Hospital, Universiti Putra Malaysia between January and December 2023 were reviewed to determine traumatic injury cases and all data were analysed descriptively. Among 422 patient medical records, 99 horses (23.0%) were reported to have sustained a total of 107 traumatic injuries. Majority of the injuries were reported to occur in the stable (26.2%), while being ridden (18.7%), and during the competition (18.7%), in which most of the cases were associated with wounds (60.7%), but the occurrence of fractures (9.3%) and injured ligaments or tendons (15.0%) were also high. Occurrence of being kicked by another horse (11.2%) and penetration by sharp objects (11.2%) were the most common mechanisms of injury, whereby forelimbs (38.8%) and hindlimbs (29.8%) were injured the most, commonly at the distal aspect. This data will enable horse owners and veterinarians to employ better traumatic injury–preventative strategies.

Keywords: Horse, prevalence, risk factor, traumatic injury

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E-mail addresses:
zulfitri.abra03@gmail.com (Zulfitri Naim Abdul Rahim)
noraniza@upm.edu.my (Noraniza Mohd Adzahan)
intanshameha@upm.edu.my (Intan Shameha Abdul Razak)
zunita@upm.edu.my (Zunita Zakaria)
cameliaroselan@gmail.com (Puteri Rose Camelia Roselan)
* Corresponding author

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INTRODUCTION

Traumatic injury refers to the damage or harm inflicted upon the structure or function of the body as a result of an external or internal force, encompassing both physical and chemical components (Owen et al., 2011). Traumatic incidents in horses can occur in general–purpose and sport horses. These injuries encompass a wide range of severity, from minor cuts and scrapes caused by saddle material, falls, kicks, or bites to more serious traumas that involve fractures of bone or torn ligament and tendon. In cases of catastrophic musculoskeletal injury, there is a high probability horses will be resorted to humane euthanasia to alleviate the suffering and painful condition.

Most recent research has focused particularly on injury in sport or performance horses, with special attention given to identifying the causes and risk factors that predispose to injuries in polo ponies (Inness & Morgan, 2014), dressage horses (Murray et al., 2010), jumping horses (Gibson et al., 2023), and racehorses (Crawford et al., 2021; Rouette et al., 2021). Briefly, tendon injuries were perceived as the major problem in polo ponies in which the hard ground provides an important risk factor for this incidence, followed by wounds and splint bone fracture. In racehorses, traumatic musculoskeletal injuries were the most common cause of death reported in Canada and the United Kingdom, comprising over 70.0% of racehorse fatalities (Allen et al., 2017; Physick-Sheard et al., 2018; Rosanowski et al., 2016). The high occurrence of traumatic injuries in sport horses has significant implications for training losses and wastage in horses across various disciplines (Mekete, 2022; Reilly & Bryk-Lucy, 2021). Nonetheless, general – purpose horses are also at risk of experiencing the incidence of traumatic injuries. A study in the United Kingdom has shown that 40.0% of general–purpose horses also sustain a traumatic injury (Owen et al., 2011). Sixty–two percent (62%) of the incidence occurred in the field, while 13.0% was during ridden exercise. The breed of horse, ownership duration, turn out environment, and stabling all time during spring were identified to be the factors associated with the increased risk of traumatic injury.

Despite injuries that take place during competition or being ridden, trauma in horses is also attributed to the incidence of bite and kick, which prevalence was found to be 8.0% and 21.6% in Swiss equine populations (Knubben et al., 2008). Apart from chasing, rearing, and mounting, biting and kicking are also typical horse behaviours and are associated with aggressive, threatening, submissive, and avoidance behaviour to preserve a long–lasting relationship, while some might happen by mistake as a result of exuberant excitement behaviour. Therefore, ensuring a stable group hierarchy and providing sufficient space in pasture and paddock have been identified as crucial elements in preventing these injuries (Knubben et al., 2008). Besides, trauma to horses in transport vehicles during a long journey also occurred frequently. The majority of research has focused on horses intended for human consumption that are transported by road using commercial companies, in which the incidence of injuries varies from 1.6% to 33.0% (Roy et al., 2015a, 2015b). In Australia, 45.0% of survey participants reported an incidence of traumatic injury related to both commercial and non-commercial horse transportation (Padalino et al., 2016). Additionally, a retrospective survey of injury during non-commercial transportation has reported that equine behaviour was the attributed cause of 56.0% of incidents reported and most incidents occurred during the first hour of travel (Hall et al., 2020).

Wounds, bone fractures, and injuries to ligaments as well as tendons were commonly associated with trauma in horses. Wounds at the limbs were the most frequently seen in the form of abrasions, lacerations, or incisions. A survey in New Zealand demonstrated a high occurrence of skin wounds, particularly on the distal limb, which were costly and time-consuming to manage and may lead to decreased performance, retirement, or euthanasia (Theoret et al., 2015). On the other hand, bone fractures can occur in any part of the body. Kick injuries have been reported to cause fractures of the second and fourth metacarpal and metatarsal bones, followed by bones of the skull, proximal and distal phalanx, pelvis, and tibia (Donati et al., 2018). The fracture can be either simple fractures, comminuted fractures, fissures, or depression fractures, as well as the least seen, were Salter–Harris fractures.

Despite the high occurrence of traumatic injury to the equine population worldwide, the data on traumatic injuries are still limited, particularly in Malaysia. Therefore, the aims of the study are to highlight the occurrence of equine traumatic injuries by determining the frequency and assessing the patterns associated with the incidence. These findings will provide valuable insights for the horse owners and direct the veterinary teams to offer more specific care and enable prevention strategies for the horses.

MATERIALS AND METHODS

The case records of all horses reported to the University Veterinary Hospital, Universiti Putra Malaysia were reviewed. Data from January to December 2023 were selected and analysed for the occurrence of the equine traumatic injury. Cases that were confirmed or suspected traumatic injury incident had taken place were selected. A confirmed traumatic injury was determined based on the keyword "Traumatic injury" indicated in the record and as well as based on the case history. Meanwhile, records for all horses with a suspected traumatic injury were examined and deemed eligible for inclusion in this study, in which any of the following criteria were met: the presence of one or several lacerations, incision, ulceration, or abrasion wounds, swelling of the affected region, pain upon palpation, lame upon walk or trot and fracture of a bone. Cases were excluded if there was insufficient or no clear evidence of a traumatic injury based on the case report. Case information was collected detailing the date of occurrence, signalment of the horse (breed, sex, age, uses), aetiology of injury, and physical examination findings. Additional information regarding the cases was obtained through verbal communication with the veterinarian responsible for the cases.

Data were entered and managed in Microsoft Excel 2021 ((Microsoft Corporation) and IBM SPSS Statistics version 27 software (IBM Corporation) was utilized to analyse the data and determine the frequency distribution. The analysed data were reported as frequencies and percentages. The distribution of traumatic injury was divided into groups in accordance with the risk factors. Outcomes of each aspect were evaluated and were expressed as a percentage of the total number of outcomes from all aspects by using tables and charts.

RESULTS

A comprehensive analysis of 422 patient medical records yielded a total of 107 traumatic injury cases reported from 99 horses (23.0%) from January until December 2023 as illustrated in Figure 1. Among these, 8 horses sustained multiple injuries, whereas the remaining 91 horses were reported to have suffered only a single injury each. The average number of cases that occurred was 9 cases in a month, which ranged from a minimum of 6 cases seen in February, March, October, and December to the highest of 15 cases reported in June.

Table 1 shows the number and percentage of traumatic injury according to sex, age, breed and horse's uses classifications. Of all horses examined, there were comparable number of mare and geldings recorded with small portion of stallion and filly. Among them, there were a higher number of adults reported with injuries compared to foal and geriatric horse. The breed distribution comprised of Thoroughbred, Polo Ponies, Warmblood, Arabian, Criollo, Friesian and local pony. Most horses were used for sport and companionship. However, in 15 horses, there was no information regarding their uses at the time of injury reported.

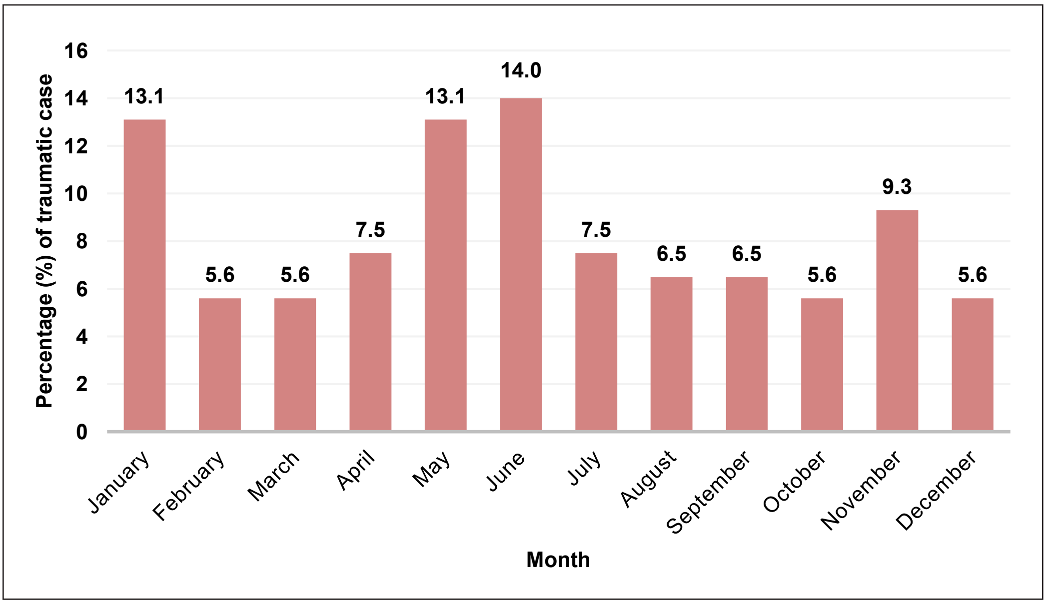

Figure 1. Monthly distribution of traumatic injury cases in horses, from a total of 107 cases, presented to the University Veterinary Hospital, Universiti Putra Malaysia, from January to December 2023

The locations of traumatic injury incident took place are shown in Table 2. The occurrence was mostly reported to occur at the stable or stall. This was followed by injuries that occurred during competition, as well as during ridden for training and leisure rides, which had similar occurrences in this study. Traumatic injury also takes place when the horse is released into the paddock. Rarely, traumatic injury was also reported to occur during transportation. However, in two cases, the location of the injury was not documented in the medical record.

Table 3 displays the causes of traumatic injury sustained by the horses over the year. The most frequent causes of injury were a kick from another horse and also penetration by

Table 1
Distribution of horses reported with traumatic injury to University Veterinary Hospital, Universiti Putra Malaysia according to sex, age, breed and uses from January to December 2023

Signalments	Frequency	Percentage (%)	Confidence Interval (95%)
Sex			
Mare	50	50.5	(0.41, 0.60)
Gelding	46	46.5	(0.37, 0.56)
Stallion	2	2.0	(0.00, 0.05)
Filly	1	1.0	(0.00, 0.03)
Total	99	100.0	
Age			
Foal	1	1.0	(0.00, 0.03)
Adult	77	77.8	(0.70, 0.86)
Geriatric	21	21.2	(0.13, 0.29)
Total	99	100.0	
Breed			
Thoroughbred	35	35.4	(0.26, 0.45)
Warmblood	16	16.2	(0.09, 0.23)
Arabian	11	11.1	(0.05, 0.17)
Polo ponies	32	32.3	(0.23, 0.42)
Criollo	1	1.0	(0.00, 0.03)
Friesian	1	1.0	(0.00, 0.03)
Local pony	1	1.0	(0.00, 0.03)
Unknown	2	2.0	(0.00, 0.05)
Total	99	100.0	
Uses			
Dressage and show jumping	23	23.2	(0.15, 0.32)
Endurance	19	19.2	(0.11, 0.27)
Polo match	27	27.3	(0.18, 0.36)
Pleasure	10	10.1	(0.04, 0.16)
Patrol	6	6.1	(0.01, 0.11)
Unknown	15	15.2	(0.08, 0.22)
Total	99	100.0	

sharp objects. Other causes involved falling or stumbling, crashes and hits that occurred during polo matches, and also improper saddle or bit used. Some horses also exhibited self–inflicted injury. This self-mutilation encompasses behaviours such as biting, stomping, kicking, rubbing, and lunging into objects. Wounded during transportation occurred rarely. However, about half of the cases, the cause of injury could not be determined.

Table 4 presents the various types of traumatic injuries sustained by horses that were presented to the hospital throughout the year 2023. Of all the trauma cases, injury presented with single or multiple cutaneous wounds contributes to the highest cases reported to the hospital. This wound includes laceration, abrasion, or incision wounds that involve any parts of the body. Injury toward the ligament and tendon accounted for 15.0% of the cases, particularly involving the ligament and tendon of the limbs. This was followed by

Table 2
Distribution of location of traumatic injury incident took place in horses presented to University Veterinary Hospital, Universiti Putra Malaysia from January to December 2023

Location	Frequency	Percentage (%)	Confidence Interval (95%)
Stable or stall	28	26.2	(0.18, 0.34)
Competition	20	18.7	(0.11, 0.26)
Ridden	20	18.7	(0.11, 0.26)
Paddock	9	8.4	(0.03, 0.14)
Transportation	2	1.9	(0.00, 0.10)
Not mentioned	28	26.2	(0.18, 0.34)
Total	107	100.0	

Note. Out of a total of 99 horses, 8 horses sustained two traumatic injuries, while the remaining 91 horses sustained a single traumatic injury

Table 3
Distribution of the causes of traumatic injury in horses presented to University Veterinary Hospital, Universiti Putra Malaysia from January to December 2023

Causes of traumatic injury	Frequency	Percentage (%)	Confidence Interval (95%)
Kick	12	11.2	(0.05, 0.17)
Stumble/ Fall	11	10.3	(0.05, 0.16)
Improper bit/ saddle	7	6.5	(0.02, 0.11)
Penetration by a sharp object	12	11.2	(0.05, 0.17)
Crash and hit during polo game	9	8.4	(0.03, 0.14)
Transportation	2	1.9	(0.00, 0.10)
Self-inflicted	7	6.5	(0.02, 0.11)
Not mentioned	47	43.9	(0.35, 0.53)
Total	107	100.0	

Note. Out of a total of 99 horses, 8 horses sustained two traumatic injuries, while the remaining 91 horses sustained a single traumatic injury fractures which mostly occur to the splint bone, where the lateral aspect of the limbs was injured in approximately 86.0% and the medial aspect in 14.0% of the cases. Rarely there were also fractures toward the sesamoid bone, nasal bridge, and tooth. Trauma to the eye attributed to the 8.4% of the cases reported. The horses were also suffering from puncture wounds which resulted from nail penetration of the sole or other penetration of muscle by sharp objects. Infrequently, traumatic incidents occurred without visible physical injury, although horses exhibited signs of inflammation and recumbency.

The occurrences of injuries on various body parts showed significant variation as demonstrated in Table 5. The proportion of injuries on the limb was the highest, in which the injury to the forelimb was significantly more than those that occurred to the hindlimb. Injury to the head region was lesser than the limb, which was attributed to the eye, mouth, ear, and nose, respectively. The traumatic injuries also inflicted the hoof, trunk, chest, and hindquarters. The genital and neck regions exhibited the lowest frequency of distribution.

Table 4
Proportion of traumatic injuries by type in horses presented to University Veterinary Hospital, Universiti Putra Malaysia from January to December 2023

Type of traumatic injury	Frequency	Percentage (%)	Confidence Interval (95%)
Single wound	44	41.1	(0.32, 0.50)
Multiple wounds	21	19.6	(0.12, 0.27)
Fracture	10	9.3	(0.04, 0.15)
Ligament/ tendon injury	16	15.0	(0.08, 0.22)
Eye trauma	9	8.4	(0.03, 0.14)
Hoof and muscle penetration/ puncture	5	4.7	(0.01, 0.09)
No visible injury*	2	1.9	(0.00, 0.10)
Total	107	100.0	

Note. Out of a total of 99 horses, 8 horses sustained two traumatic injuries, while the remaining 91 horses sustained a single traumatic injury. * = Horses were reported to have experienced traumatic incidents exhibited signs of inflammation and recumbency, with an absence of visible external or internal injuries

Table 5
Distribution of the traumatic injuries relative to anatomical location in horses presented to University Veterinary Hospital, Universiti Putra Malaysia from January to December 2023

Anatomical location distribution	Frequency	Percentage (%)	Confidence Interval (95%)
Head			
Eyes	9	7.4	(0.03, 0.12)
Mouth	7	5.8	(0.02, 0.10)
Ear	1	0.8	(0.00, 0.03)
Nose	1	0.8	(0.00, 0.03)
Neck	1	0.8	(0.00, 0.03)

Table 5 *(continue)*

Anatomical location distribution	Frequency	Percentage (%)	Confidence Interval (95%)
Chest	3	2.5	(0.00, 0.05)
Trunk	6	5.0	(0.01, 0.09)
Hindquarters	3	2.5	(0.00, 0.05)
Genital	1	0.8	(0.00, 0.03)
Forelimb	47	38.8	(0.30, 0.47)
Hindlimb	36	29.8	(0.21, 0.38)
Hoof	6	5.0	(0.01, 0.09)
Total	121	100.0	

Note. The total figure indicates to horses that have experienced one or two injuries, and also horses with multiple wounds presented with injuries at multiple body locations

DISCUSSION

This retrospective study presents a comprehensive description of the frequency and patterns of injuries experienced by a subpopulation of horses in Peninsular Malaysia. Overall, all cases reported to the veterinary teaching hospital within 12–month period showed the occurrence of horses with traumatic injuries was 23.0%. The figure is lower compared to the 40.0% reported by a previous study in the United Kingdom (Owen et al., 2011). This difference could be attributed to the data collection approach used, as this study collected information on the injury incidents reported to the hospital and which were attended by the veterinarian. Meanwhile previous study by Owen et al. (2011) used a questionnaire survey from horse owners who may have reported minor injuries that may not have been attended by a veterinarian. Additionally, a study by Satessa and Lema (2014) reported an overall injury occurrence of 45.5% in working equines, which is higher than the occurrence observed in this study. The nature of these animals as working equines may increase their exposure to injury due to the physical demands and environmental factors associated with their labor. However, our occurrence appeared to agree with the survey studies in Australia and New Zealand that reported their occurrence as 22.5% and 25.0% respectively (Sole et al., 2015; Theoret et al., 2015). Indeed, our figure may underestimate the true prevalence of traumatic injury in Malaysia as it only evaluated a small portion of the horse population in Peninsular Malaysia. Besides, there is also the possibility that the horse owners did not report the trauma incidents to the veterinarian as well as they treated the condition without veterinary assistance. Similar to situation in Australia, where horse owners are known to manage some wounds independently, which could lead to underreporting of injury cases (Sole et al., 2015). While these studies did not assess the welfare impact of this practice, it raises concerns about the effects on horse health, particularly given the need for proper veterinary care for serious injuries.

Moreover, the age and sex distribution exhibited no significant influence on the occurrence of traumatic injury in the horses. However, the breed of horse and its use appear to have an impact on raising the risk of exposure to a traumatic incident. In this study, horses engaged in competitive activities experienced a higher frequency of sustaining injuries. The endurance competition mostly featured by Thoroughbred and Arabian horses, while polo matches involved Polo ponies, and Warmbloods were utilized in dressage and show jumping events. As endurance is considered a strenuous exercise, it is noteworthy that horses were exposed to sharp object penetration on the track, along with falls or stumbles due to fatigue. The speed, inexperienced rider, previous injuries of the horses, track and other unmeasured factors may also co–influence the risk for traumatic injury during an endurance race (Nagy et al., 2013). Moreover, given the nature of the polo game that requires the ponies to sprint at maximum speed, turn abruptly, make sudden stops, retain balance when riders shift their weight significantly off–centre, and even collide with other horses, the potential for injuries is consistently high for the ponies as well as for the players. This discovery aligns with the findings of a previous study which also identified tendon injuries, wounds, and splint bone fractures as the most often reported injuries in polo ponies (Inness & Morgan, 2014). Additionally, a significant number of show jumping and dressage horses also encounter difficulties related to their ligaments and tendons. This supports previously described clinical findings that demonstrated a high risk of forelimb superficial digital flexor tendon (SDFT) injury and distal deep digital flexor tendon (DDFT) injury in show jumping horses and hindlimb suspensory ligament injury in dressage horses (Murray et al., 2006; Tranquille et al., 2024). This could be attributed to the repetitive loading during jumping and considerable strain on landing, which relatively cause a great strain on the show jumping horse (Murray et al., 2006; Sousa et al., 2017). Meanwhile, the training regime and surface–related factors normally predisposed the dressage horse to this injury (Murray et al., 2006; 2010). Hence, performance horses are at greater risk of sustaining traumatic injury compared to general–purpose horses.

Despite the high number of horses used for sport, many horses were injured while in the stable compared to during competition, where the incident took place at the stable compound or in the individual stalls. Other than mechanical injury, the individual housing practice possibly induces abnormal behaviour in the horses which leads to self–inflicted injury. This study discovered that some horses had self–mutilation behaviours, in which there was a tendency to bite their bodies and as well as to scratch the stall wall. This self–inflicted injury can be caused by firstly normal behavioural response to continuous or intermittent physical discomfort, secondly by self–directed intermale aggression, or thirdly by stereotypic activity, for example nipping at various areas of the body in a relatively invariant pattern, stomping, or kicking rhythmically against an object (McDonnell, 2008; Winskill et al., 1995). Additionally, horses that were socially confined also may be injured or injure other horses when they are released to stay in the group, particularly in the paddock (Knubben et al., 2008). These horses were predisposed to demonstrating abnormal behaviours to redirect sexual behaviour, as a defensive mechanism, or to establish hierarchy within a group (Knubben et al., 2008; McGreevy, 2004). These abnormal or exaggerated normal behaviours may be likely to increase the risk of injury to horses within the group. Furthermore, the horse also was injured when ridden, both for training and leisure. One of the issues was caused by poor saddle or bit used. Poorly fitted saddle will cause wounds, particularly at the wither and loin region. An ill-fitting saddle can hinder the horse's thoracolumbar function, leading to stiffness in that area, which may contribute to back pain for the rider (Dyson et al., 2015). Similarly, a saddle that does not fit the rider properly can affect their position and balance, resulting in back or hip pain. Identification of an ill-fitting saddle involves inspecting it both off and on the horse without and with the rider, while observing horse movement. Examine for uneven flocking, ensure the gullet is appropriately sized, and check for complete tree coverage to avoid pressure on the spine. A saddle that tips forward or backward or has uneven panel contact can cause pain and restrict the horse's movement. Indeed, correct saddle fit for horse and rider is an important equine welfare issue. Besides, the bits used might also wound the oral region. The bit induces pressure on the sensitive structures of the horse mouth, which include the lip commissures, buccal mucosa, tongue, and the bars of the lower jaw, and in certain bit types, it also causes pressure to the hard palate and the base of the second premolars (Anttila et al., 2022; Manfredi et al., 2005a; 2005b; 2009). However, an ill-fitting bit can potentially put excessive pressure, pinching or rubbing of oral tissues or limit the movement of the tongue, which eventually causes oral trauma and thus pain for the horse (Björnsdóttir et al., 2014; Mata et al., 2015; Tuomola et al., 2019). Hence, a significant number of traumatic injuries also happen in stables and during ridden due to factors like poor housing practices and ill-fitting equipment, thus it is vital to monitor and understand horses' behaviour as well as to ensure the proper choice of saddle and bits to minimize the traumatic injury incidents.

In addition, the cause of most injuries was not determined as it was not mentioned in the patient record as well as owners were not present at the time of injury. However, in this study, kicking by another horse and penetration by a sharp object were the most common mechanisms of injury to happen. There were significantly larger proportion of Thoroughbreds were victims of kick injuries than were other breeds, which is a finding in line with a previous study that revealed Thoroughbreds and Thoroughbred crossbreds as more likely to be victims of kick wounds (Schroeder et al., 2013). This study also found that kick injuries most take place when a group of horses are let into the paddock together, this is a similar scenario to a previous study that demonstrated that kick incidents occurred on pasture (Derungs et al., 2004). As an important aspect of equine behaviour, kicking serves as a defensive mechanism and a means of establishing group hierarchy, particularly occurring when dominant or socially confined horses are placed together (Knubben et al., 2008; McGreevy, 2004). Penetration by sharp objects occurred mostly at the sole of the hoof, and caused laceration of the corneal of the eye. The nails including the horseshoe nail were the main cause of the punctured sole. Meanwhile, laceration of the corneal normally resulted from stable doors, fence posts, trees, and sand. Both penetration of the sole and corneal by a sharp object can be deadly if it is involved with vital structure and normally is associated with the introduction of pathogenic microorganisms (Burba, 2013; Lazareva et al., 2022). Stumble and fall took place during training or competition, and even at stable. This is contributed by the uneven terrain or slippery surfaces that make horses difficult to maintain their footing. Horses undergo strenuous also may experience fatigue or limb and hoof issues which lead them to a lack of coordination and increased risk of stumbling. Moreover, the traumatic injury also occurred due to the crash and hit during a polo match, which focused more on the polo horses. Despite, the high number of wound cases determined in this study, polo horses are always at risk for tendon injuries due to their nature and need for speed, agility and constant exposure to stress during the match (Inness & Morgan, 2014; Schumacher & Gehlen, 2024). Transportation–associated injuries usually occurred during vehicle movement, and less frequently during unloading, loading and while stationary, in relation to behaviours such as scrambling and panicking (Riley et al., 2016). It has also been reported that mechanical failure of a trailer or truck, driver errors, as well as traffic and road condition contribute to injury during the transportation (Riley et al., 2016).

The forelimb and hindlimb of horses experienced the highest number of injuries, with the distal portion of the limb being the most commonly affected area. Wounds were the most prevalent type of injury, followed by problems with ligament and tendon and splint bone fracture. Among the causes of wounds to the limb were related to the penetration by sharp objects, stumbles and falls, kicks as well the injury during polo matches. The lower limb is subjected to injury due to its limited soft tissue, thus resulting in inadequate protection compared to the upper limb and trunk (Kayode, 2017). Wounds that involve the distal aspect of the limb are frequently more problematic due to their closeness to the ground makes them more likely to become contaminated and infected (Jørgensen et al., 2021; Kayode, 2017). Moreover, the head region is also exposed to injury, particularly the muzzle and eye. As mentioned above, the high incidence of injury at the muzzle or generally oral region is normally correlate with the poor choice of bits. It is vital to identify the correct bit size in accordance with each horse's oral dimension. Since, the oral dimensions vary by age, sex, and breed, measuring oral dimensions as part of routine dental examination aids in choosing a bit mouthpiece size that fits to avoid discomfort (Anttila et al., 2022). There is a notable correlation between the eye injuries sustained with performance horses which may be related to penetrating objects such as sand for racehorses and polo sticks in polo ponies, however, there was also exposure of potentially hazardous in the stable, such as feed troughs, water buckets, and hay racks. A study showed that horses kept in stable, sustained more ocular disease compared to the horses kept primarily at pasture (Ludwig et al., 2025). Even though this study reported more findings on ulcerative eye lesions, the horses are also always at risk to sustain the non–ulcerative eye lesions that able to lead to detrimental conditions such as fungal stromal abscess formation, cataracts and corneal perforations.

CONCLUSION

In conclusion, this present study has demonstrated that there is significant occurrence of traumatic injury in the subpopulation of horses in Peninsular Malaysia. It was also shown that the majority of injuries were noted in the horses that participated in competition, however general–purpose horses are also predisposed to traumatic injuries, and the most frequent causes of injury were being kicked by another horse and being penetrated by a sharp object. Most of the cases were presented with wounds and the forelimb and hindlimb were the body parts implicated by most traumatic injuries, which were expensive and time–consuming to treat and may lead to reduced performance, retirement, or euthanasia. Therefore, these findings highlight the need for a better preventive measure, improved management practices, and stronger safety protocols**.** Ultimately, a comprehensive understanding of these injury patterns will promote the overall health and welfare of horses. This proactive approach can significantly reduce the incidence of traumatic injuries in equine populations.

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Short Communication

First Report and Yield Reduction of Emerging Yellow Spot Disease on Melon (*Cucumis melo*) Caused by Melon Yellow Spot Virus (MYSV) in Indonesia

Sedyo Hartono[1]*, Argawi Kandito[2] and Achmadi Priyatmojo[1]

[1]*Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Jalan Flora no. 1, Bulaksumur, Yogyakarta 55281, Indonesia*

[2]*Agrotechnology Program, Faculty of Agriculture, Universitas PGRI Yogyakarta, Jalan Sonosewu Baru, Ngestiharjo, Bantul, Yogyakarta 55182, Indonesia*

ABSTRACT

Typical symptoms of melon yellow spot disease include leaf curling, mosaic, chlorotic spots, fruit discoloration, and cracking, which are constantly found in melon greenhouses in Indonesia. These symptoms lead to considerable losses, reducing fruit weight by up to 66.67% and lowering the Brix score, making the fruit unmarketable. RT-PCR targeting the N-gene of MYSV showed all samples were positively infected. Bioinformatics analysis revealed that Asian isolates of MYSV are highly identical and share a common ancestor, highlighting MYSV as an emerging disease to melon production across Asia.

Keywords: Emerging disease, melon, MYSV, RT-PCR, yield reduction

INTRODUCTION

Melon (*Cucumis melo*) is one of the important cucurbit crops in the world. About 70% of melon productions are in Asia. Indonesia is one of the melon producers in Asia with annual production reaching up to 118,696 tonnes (BPS-Statistics Indonesia, 2022). In the last three years, melon production in Indonesia has decreased by more than 14% due to the high prevalence of plant diseases caused by various pathogens. Viruses account for the most anticipated pathogens for melon due to their rapid transmission and significant losses of up to 100%. There are several virus genera known to be associated with melon diseases in Indonesia, namely *Potyvirus,*

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E-mail addresses:
sedyohartono@ugm.ac.id (Sedyo Hartono)
argawikandito@gmail.com (Argawi Kandito)
priyatmojo@ugm.ac.id (Achmadi Priyatmojo)
* Corresponding author

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Tobamovirus, Cucumovirus, Begomovirus, *Comovirus*, and *Orthotospovirus* (Adachi-Fukunaga et al., 2020; McLeish et al., 2022).

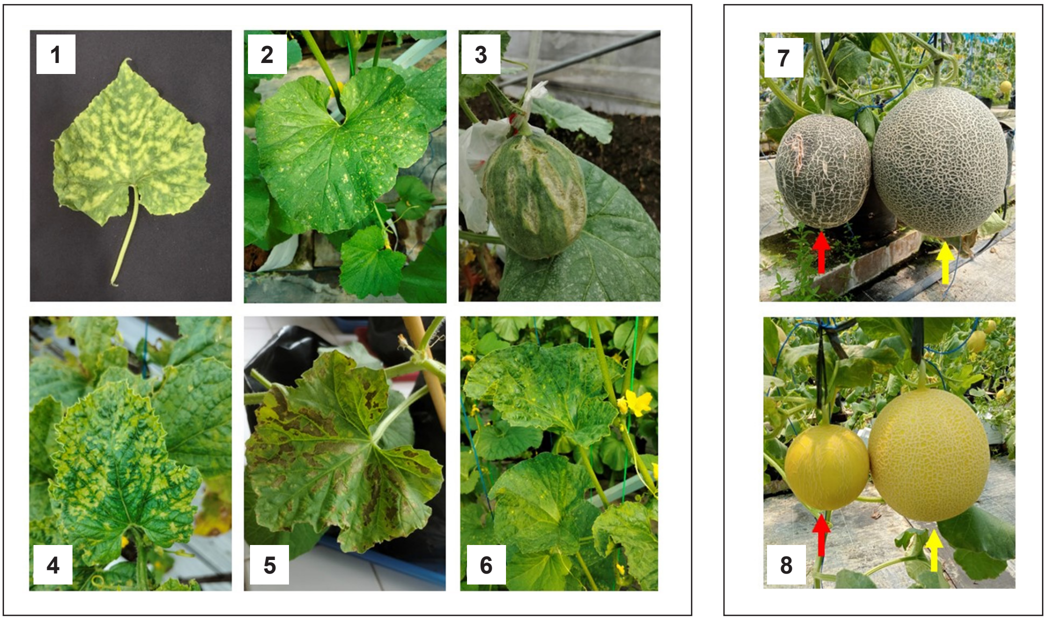
Yellow spot disease is currently emerging in Asia and is becoming a major threat to melon cultivation. It is caused by melon yellow spot virus (MYSV) (Chen et al., 2010). MYSV is a member of the *Orthotospovirus* genus. It was known persistently transmitted by thrips and was first reported in Japan (Adachi-Fukunaga et al., 2020; Chakraborty et al., 2018; Kato et al., 2000). Later, MYSV was found in several countries including China (Gu et al., 2012; Sun et al., 2020), Taiwan (Peng et al., 2011), Thailand (Chiemsombat et al., 2008; Supakitthanakorn et al., 2018), Ecuador (Quito-Avila et al., 2014), and India (Pradeep et al., 2024). The symptoms of MYSV infection are chlorotic spot, mosaic, leaf curl, fruit discoloration, and fruit cracking resulting in production failure and total economic loss due to unmarketable products. Compared to established viral infections like Begomovirus, Orthotospovirus infections have the potential to become epidemics in the future. This is due to the wide host range, high genetic diversity, persistent transmission by thrips, and the lack of resistant sources (Pradeep et al., 2024).

During the 2021-2023 survey in several melon production greenhouses in Indonesia, we constantly found melon plants exhibiting symptoms including leaf curling, leaf mosaic, chlorotic spots, necrotic spots, fruit discoloration, and fruit cracking with incidence reaching up to 90% in melon population (Figure 1). These symptoms appear simultaneously with the high thrips (*Thrips parvispinus*) population, resembling typical orthotospovirus infections. These symptoms followed by a high thrips population have never been found before in Indonesia. Considering the significant importance of the disease for melon cultivation and developing management strategies, this research aimed to determine the causal agent of the disease and evaluate the damage to melon cultivation. We carried out molecular detection targeting several common viruses on melon including *Tobamovirus, Potyvirus, Begomovirus*, and MYSV. However, of all tests carried out, only MYSV detection showed positive results, indicating that the yellow spot disease on melon is caused by MYSV. This finding suggests that yellow spot disease caused by MYSV is a novel pathogen in Indonesia and poses a serious threat to melon cultivation.

MATERIALS AND METHOD

Sample Collection

Melon leaves showing yellow spot symptoms were collected from four greenhouse locations in Central Java: Solo, Bergas, Yogyakarta, and Tegal. Leaves of the diseased plants were collected in purposive sampling. Samples collected from the plant showed leaf mosaic, leaf spot, leaf chlorosis, and leaf necrotic spot in the early generative stage. The early generative stage is the critical time of plants from thrips infestation. Samples were documented, stored dry, and subsequently used for RT-PCR.



(a) (b)

Figure 1. (a) Symptom variations of MYSV in melon plants; and (b) size comparison between fruits. Symptom variations could be a leaf mosaic (1), yellow spot (2), fruit cracking (3), leaf curling and mosaic (4), leaf necrotic (5), or leaf cupping (6). Fruit from diseased plants (7 & 8 red arrows) are smaller, cracked, and might have uneven nets compared to the healthy one (7 & 8 yellow arrow)

Field Observation and Damage Evaluation

Two greenhouses located in Solo and Bergas were utilized to model yield losses in melon due to disease. Each greenhouse housed an estimated 3,000-4,000 plants. Disease impact was assessed by evaluating several parameters: disease incidence, severity, fruit weight loss, and fruit Brix. Disease incidence was determined by calculating the proportion of diseased plants within the total plant population in each greenhouse. Disease severity was assessed by quantifying the number of symptomatic leaves on each plant relative to the total number of leaves. Fruit weight loss was determined by comparing the weight of fruits from diseased plants to those from healthy plants. Finally, fruit Brix was measured using a refractometer.

RNA Extraction and RT-PCR

Samples were subjected to RT-PCR. Total RNA extraction was performed using the Total RNA Mini Kit (Geneaid, Taiwan) according to the manufacturer's instructions. cDNA synthesis was performed using a Revertra-ace cDNA synthesis kit (Toyobo, Japan) with an n-hexamer primer. cDNA was subsequently used as a PCR template. PCR was performed in a total 50 µL reaction consisting of 25 µL MyTaq HS Red Mix 2X (Meridian, USA), 2 µL each forward and reverse primers (10 pmol/µL), 19 µL nuclease-free water, and 2 µL cDNA (10 ng/µL) as template. The primers used to target the nucleocapsid (N) gene of MYSV (Charlermroj et al., 2017). The PCR conditions were pre-denaturation at 95°C for 3 minutes followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. PCR products were visualized by 1% agarose electrophoresis and subjected to bidirectional Sanger sequencing.

Bioinformatics Analysis

Nucleotide sequence data was analysed using BLAST (https://blast.ncbi.nlm.nih.goc/Blast.cgi/) and subsequently deposited in Genbank (Accession No. OR405986-0R405989). Nucleotide alignment was performed using ClustalW. The phylogenetic tree was constructed using MEGA 7 with Neighbor-Joining Method and 1000 bootstraps replication (Kumar et al., 2016).

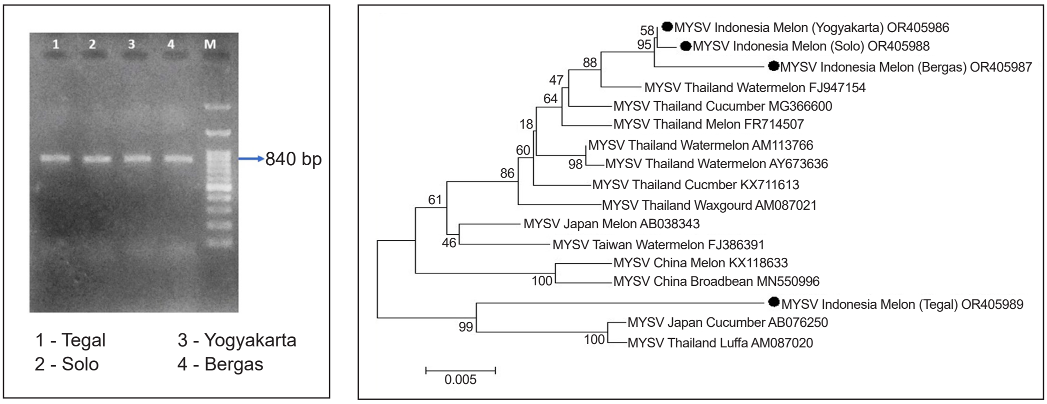
RESULTS

Field observations revealed characteristic MYSV symptoms including leaf cupping, dark green spots on leaves, and vein yellowing. These symptoms specifically appeared during the early generative phase, from the onset of flowering until fruit set (Figure 1). The early generative phase, particularly during flower formation, is a highly preferred phase for thrips (Ren et al., 2020).

The data showed disease incidence can reach up to 85%, indicating that MYSV is prevalent in these locations. Comparison between healthy plants vs diseased plants showed that the disease caused significant weight losses up to 66,67% and significantly lower average Brix score (9 vs 13 on healthy plants) (Table 1). The lower weight and Brix score on diseased plants consequently caused the fruit not to meet the market standard. Moreover, it resulted in total economic losses.

Table 1
Field observation data on two greenhouses

Greenhouses	Population (plants)	Status	Severity (%)	Incidence (%)	Fruit Weight (gr)	Brix
Solo	4000	Healthy	0	0	1500	13
		Diseased	50	85	500	9
Total loss percentage			0	0	66,67%	30,71%
Bergas	3500	Healthy	0	0	1350	13
		Diseased	35	70	550	9
Total loss percentage			0	0	59,26%	30,71%



(a) (b)

Figure 2. (a) PCR Visualization; and (b) phylogenetic diagram of samples obtained from Tegal (1), Solo (2), Yogyakarta (3), and Bergas (4). The phylogenetic tree of partial N-gene of MYSV using the Neighbor-Joining method with 1000 bootstraps replication showed MYSV isolates in Asia are closely related to each other

All samples tested for MYSV resulted in expected bands of approximately 840 bp (Figure 2). BLASTn search results of nucleotide sequences showed 96-99% identity with MYSV from Japan and Thailand from various host sources. A phylogenetic tree constructed from partial N-gene of MYSV showed Asian isolates are clustered in a single branch indicating these isolates share a common ancestor (Figure 2). These results indicate that the MYSV isolates spread in Asia are closely related. To our knowledge, this is the first report of MYSV in Indonesia.

DISCUSSION

The existence of MYSV in Indonesia poses a serious threat not only to melon cultivation but also to other cucurbitaceae cultivation in general. MYSV was also known to infect several crops including cucumber, watermelon, pumpkin, balsam pear, and chili pepper (Sunpapao, 2012; Supakitthanakorn et al., 2018; Takeuchi et al., 2009). This shows that the potential for disease caused by MYSV in horticultural crops is quite large in the future.

Our study indicates that the MYSV detected in Indonesian melons likely shares a common ancestor with MYSV isolates found in other Asian hosts, suggesting potential cross-transmission among various plant species, including weeds (Yamasaki et al., 2012). Further distribution of MYSV might exacerbated by common agricultural practices in Indonesia, particularly open-field intercropping. Open-field systems allow the movement of insect vectors between primary crops and weeds, which can serve as alternative virus hosts. While MYSV is primarily transmitted by *Thrips palmi* (melon thrips), it is hypothesized that MYSV might also be transmitted by other thrips species, such as *T. javanicus, T. tabaci,* or *T. parvispinus*. *Thrips parvispinus* has become a dominant species in Indonesia, replacing *T. palmi* (Murai et al., 2010; Sartiami & Mound, 2013).

The MYSV transmission is exclusively facilitated by thrips in a persistent manner. Thrips is also known as cosmopolitan insects with a broad host range. The widespread presence of thrips and their ability to transmit MYSV across different plant species underscore the need for comprehensive management strategies to control the viral spread. (Chakraborty et al., 2018; Peng et al., 2011).

The existence of MYSV as a novel pathogen in Indonesia adds to the long list of diseases in Cucurbitaceae. Thrips as the exclusive vector of MYSV have a short life cycle, reproduce by parthenogenesis, and are quickly resistant to insecticides (Wakil et al., 2023). Up to now, thrips are known resistant to classes of insecticides including organochlorines, organophosphates, carbamates, pyrethroids, and spinosyn (Negash et al., 2020). Until now, there are no known effective chemical pesticides to control thrips (Gao et al., 2012). Meanwhile, control measures using traps, net houses, and biological agents are so far not known to have a significant impact. Therefore, thrips control must be carried out with combined measures in an IPM framework.

To effectively control the spread of MYSV, a comprehensive approach is essential, including the eradication of diseased plants, vector management, and greenhouse sanitation. Thrips, as the primary vector of MYSV, play a crucial role in the virus transmission, thus, the control of thrips is the key to disease management. Simultaneously, the eradication of infected plants minimizes the inoculum source, preventing further viral propagation. Implementing strict greenhouse sanitation practices, such as regular cleaning and disinfection, reduces the risk of MYSV transmission by eliminating potential breeding grounds for thrips and other pests.

MYSV is expected to emerge as a significant pathogen of cucurbits shortly due to its persistent transmission by thrips and its ability to infect a broad range of host plants. The absence of commercially available melon cultivars with resistance to tospoviruses further underscores the urgent need to develop resistant varieties. This challenge is particularly critical for horticultural breeding programs focused on cucurbits. The insights gained from this research are expected to play a key role in shaping effective MYSV control strategies and guiding the development of tospovirus-resistant melon cultivars, thereby safeguarding future melon production. Further research should focus on the transmission mode and ecological aspect of virus-vector relationships.

CONCLUSION

The occurrence of yellow spot disease in melon caused by MYSV poses a serious threat to melon cultivation in Indonesia. The disease caused significant losses in fruit weight and decreased fruit quality, resulting in unmarketable fruits and total economic loss. To the best of our knowledge, this is the first report of MYSV and its losses in Indonesia.

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Effects of Eggshells as Organic Fertilizer on Growth of *Brassica juncea* (Mustard Green)

Ameera Abdul Reeza

Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA, Kampus Jasin, Cawangan Melaka, 77300 Merlimau, Melaka, Malaysia

ABSTRACT

Eggshell can be very useful as an organic fertilizer in agricultural practices as the main substance found in eggshells are calcium carbonate as well as other micronutrients which might enhance plant growth. The aim of this study was to evaluate the effects of different amounts of eggshells application toward *Brassica juncea* growth. There were 5 treatments with 3 replicates arranged in a completely randomized design (CRD) where treatments are arranged as T1 (control) without eggshell, 1 g of eggshell (T2), 5 g of eggshell (T3), 10 g of eggshell (T4) and 15 g of eggshell (T5) application. Results showed significant differences in crop growth with 11-36% increase in height, 11-70% increase in number of leaves and 26-171% increase in leaves when compared with control, where the most pronounced growth was observed in T5 (15 g of eggshell application). Significant increase was also observed in clay loam soil pH from pH 5.5 to pH 7.52 (T5) and 12-75% increase calcium content in soil but no significant difference was found in plant nutrient contents among treatments. It can be concluded that the addition of eggshells may improve the growth of *B. juncea* as well as soil pH and available calcium content.

Keywords: Brassica juncea, calcium, eggshell, growth, nutrient content, soil pH

INTRODUCTION

Fruits and vegetables are the commodity with high commercial value and have many benefits where vegetables are an important crop dietary component in supplying vitamins and fibres. *Brassica juncea*, from family of Brassicaceae commonly known as mustard green, brown mustard and Chinese mustard is originated from India. This species is a fast-growing vegetable rich in many nutritional compounds including

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E-mail address:
ameera@uitm.edu.my

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antioxidants, proteins and minerals. Imported leafy vegetables prices specifically Brassica are still high even after COVID-19 pandemic had subsided where the price has risen up to 30% and 40% (Tan, 2021) from RM3.00 to RM9.00 due to the escalating costs of agricultural inputs, labour shortage, and irregular weather in Malaysia. Such price increment is also due to the production being lower than its consumption (Federal Agricultural Marketing Authority, 2021) leading to imports of Brassica to meet the demand. Hence, increasing the local production while lowering their imports is therefore perceived as a way to alleviate the deficiency of such vegetable supplies.

Hence, the high demand for *B. juncea* in the market urges farmers to make extensive attempts in increasing the supply of these vegetables, and generally this is done with higher fertilizer application rates that may be harmful for environment and ecosystem (Najarian et al., 2021). Currently, it is very much recommended to apply organic fertilizers instead of inorganic fertilizers due to the negative impact of inorganic fertilizers (Brankov et al., 2020). Various types of organic waste materials can be used as organic fertilizers such as the undervalued form of household waste, the eggshell (Anugrah et al., 2021).

Each day, huge quantities of eggshells are thrown away as organic waste globally. Eggshell waste disposal contributes to environmental problems where the smell of eggshells provides a site for flies and abrasiveness (Gaonkar & Chakraborty, 2016). As much as 250,000 tons of eggshell wastes are produced annually around the world (Verma et al., 2017). At the same time, the total consumption of eggs by Malaysians is 295 eggs per year, which is the highest rate among Asian countries as eggs are used in many recipes in Malaysia. Most of eggshell consist of calcium carbonate, a common form of calcium while the remaining comprised of proteins as well as other minerals. Moreover, as much as 95-97% of calcium carbonate are in the form of crystals, which are stabilized by the protein matrix inside the eggshell (Haroon et al., 2015). Although most eggshells are made of calcium carbonate, it is assumed that the protein matrix plays an integral role in egg strength. As eggshells contain calcium and other micronutrients, they are the most promising organic source of calcium where 90% of the nutrient is much easier to be absorbed than limestone or other coral sources (Radha & Karthikeyan, 2019). Eggshell contain healthy and balanced calcium attributed to the trace amount of other minerals simultaneously providing an important source of calcium for growing crops while at the same time also able to deter certain pests without the need for chemicals (Karne et al., 2023) since it consists of up to 93% calcium carbonate and other trace elements which make it an excellent source of organic fertilizer (Radha & Karthikeyan, 2019). Henceforth, this research was conducted to study the effects of different amounts of eggshells as an organic fertilizer on growth of *B. juncea* while reducing the organic wastes and promoting sustainable environmental and agricultural practices.

MATERIALS AND METHODS

Preparation of Experimental Materials

The experiment was performed in a greenhouse situated in the Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA, Jasin Campus, Malacca, Malaysia (2°13’44.9”N 102°27’20.8”E). The cultivation of *B. juncea* took about 2 months from October 2023 until November 2023. As much as fifteen (15) healthy seeds of *B. juncea* of uniform in size were sown in a transplanting tray filled with peat moss and watered twice per day. The seedlings were then transplanted with one seedling per polybag when two sets of leaves had developed which was about 14 days after sowing into polybags of size 14 inch × 14 inch. The polybags were filled with topsoil taken at a nearby field in the faculty at 0–15 cm depth of a clay loam texture from Malacca series (Typic Hapludox). As much as 10 kg of topsoil were filled in each of the polybags. The watering was done manually which was provided twice per day in the morning and evening (1 L/ polybag). On the other hand, as much as 3 kg of fresh eggshells were collected, washed, and dried. Then, the eggshells were grinded to a fine powder in a mixer grinder.

Treatment Application

Each seedling was applied with 5 g NPK Green (15:15:15) at every selected period (21 DAS and 30 DAS) while the powdered eggshell treatments were surface-applied on a bi-weekly basis (8 DAS, 21 DAS, 35 DAS and 50 DAS) for 8 weeks of growth according to the treatments shown in Table 1.

Table1
Treatments used in the study

Treatment	Description
T1	Without eggshell + 5 g NPK green fertilizer per polybag
T2	1 g eggshell + 5 g NPK green fertilizer per polybag
T3	5 g eggshell + 5 g NPK green fertilizer per polybag
T4	10 g eggshell + 5 g NPK green fertilizer per polybag
T5	15 g eggshell + 5 g NPK green fertilizer per polybag

Experimental Design and Layout

This experiment followed a completely randomized (CRD) design with a total of 15 experimental units consisting of 5 treatments with 3 replications. CRD was chosen as a design for this study since the microclimate in the greenhouse was constant throughout the area where relative humidity was 80%, temperature throughout the greenhouse was 34 °C in the afternoon, sunshine was more than 5.5 hours/day with more than 17 MJ per sq m per day, while mean wind speed was measured at about 10 m/sec.

Data Collection

The plants were grown for 8 weeks (56 DAS) where during harvest, the *B. juncea* plants were measured in terms of plant height, number of leaves, width of leaves, soil pH, as well as soil and plant nutrient analysis. Plant height was measured from the collar of the plant at the surface of the topsoil up to the highest tip of the plant by using a measuring tape while the number of leaves per plant was measured by only counting fully open true leaves. The width of leaves was measured by determining at the widest point perpendicular to the longitudinal axis of the leaf. Soil pH was measured using a pH meter at a 1:2.5 soil-to-solution ratio (Enio et al., 2021). For soil nutrient analysis, available calcium, magnesium, and potassium were determined using ammonium acetate (NH_4OAc) (Reeza et al., 2021) while available phosphorus was determined using the method of Bray-2 (Bray & Kurtz, 1945). For plant nutrient analysis, the dry ashing method was employed (Sahrawat et al., 2002).

Statistical Analysis

Analysis of variance (ANOVA) was used to test significant differences between treatments using statistical package for social science (SPSS) (version 21) software while means of the treatments were compared using Tukey's test at $p \leq 0.05$.

RESULTS AND DISCUSSION

Crop Morpho-Physiological Traits

The height of *Brassica juncea* at 56 DAS (at harvest) significantly increased with the increasing amount of eggshell applied, where T5 with 15 g of eggshell was significantly the highest in height with increment of 36% more compared to control (T1) as displayed in Figure 1. Similar results were also found by Anugrah et al. (2021) and Casinillo et al. (2024) where the height significantly increased as the amount of eggshells applied increased. This might be attributed by the high calcium content in eggshells which is known to trigger the establishment of seed and root hairs as well as strengthening the stems resulting in stem elongation produced from repeated cell divisions. Hence, the subsequent elongation of cells produced by the apical and intercalary meristems from shoot apical meristem (Wang & Li, 2008) thus increase the plant height. Therefore, it can be deduced that the application of eggshells may have affect the height of *Brassica juncea*.

Similar to the results in plant height, the number of leaves significantly increased as treatments were increased (Figure 2) where T5 had the maximum number of leaves with an increase up to 70% compared to control in T1 (no eggshells) which significantly had the least number of leaves. According to Saragih et al. (2016), the constitution of the eggshell comprises of 97% calcium carbonate, 3% magnesium and 3% phosphorus alongside with traces of sodium, potassium, zinc, manganese, iron, and copper. The added macro and

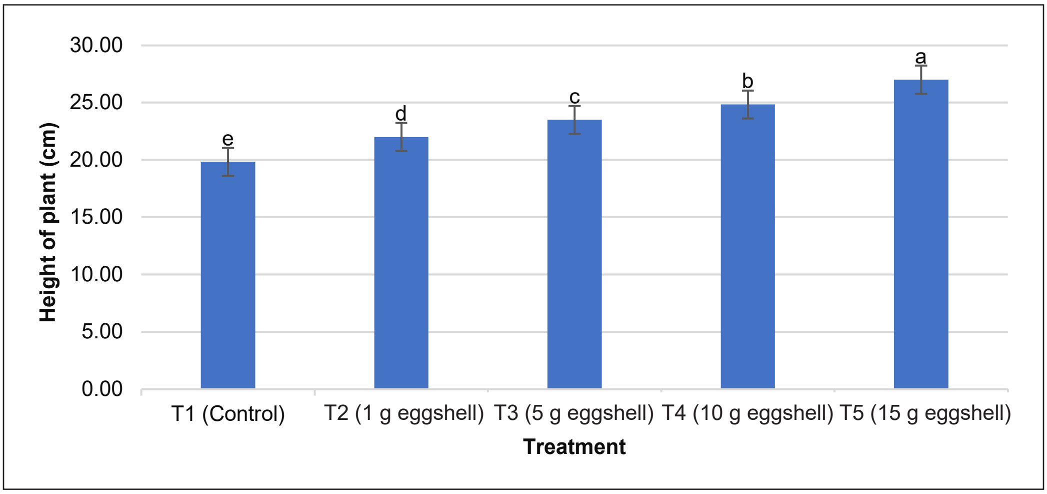

Figure 1. Mean of height of *Brassica juncea* at harvest (56 DAS). Different letters in a column is significantly different according to Tukey's test ($p \leq 0.05$)

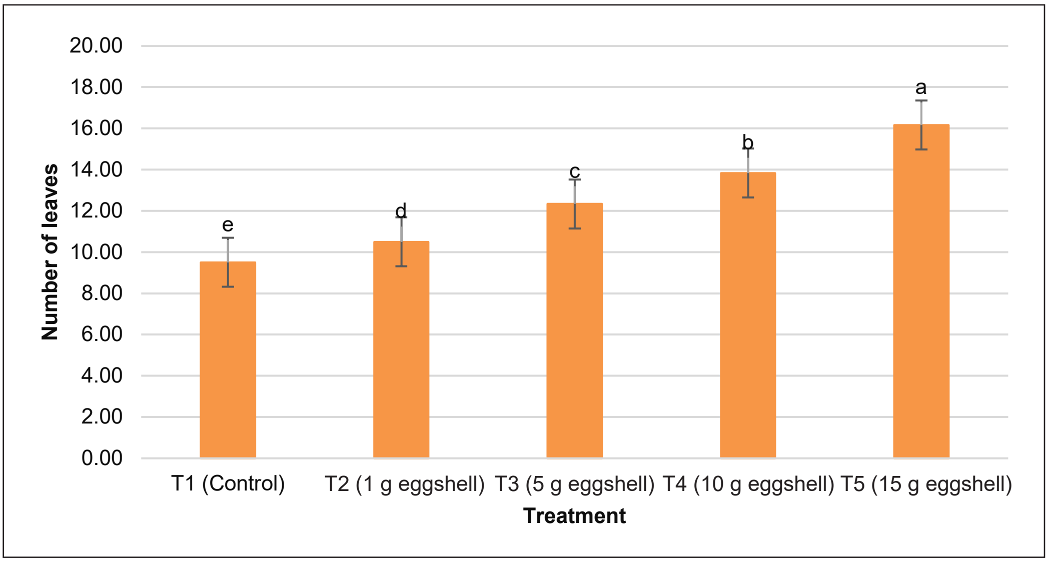

Figure 2. Mean number of leaves in *Brassica juncea* at harvest (56 DAS). Different letters in a column is significantly different according to Tukey's test ($p \leq 0.05$)

micronutrients may have contributed to increasing stem elongation as well as the number of leaves. This result is also consistent with other reports (Muyassir et al., 2022; Radha & Kartikeyan, 2019) whereby higher amounts of eggshells increase the number of leaves in *Brassica juncea* and *Phaseolus vulgaris*.

Figure 3 showed that the width of leaves significantly increased with the increasing amount of eggshells applied with increment from 26% (T2) up to 171% in T5. Such observation is similar with the previous parameters of plant height and number of leaves in *Brassica juncea*. The width of leaves for *Brassica juncea* showed that T5 significantly had the highest width while T1 significantly had the lowest width of leaves. Similar finding was also reported by Radha and Kartikeyan (2019) where the leaf area as well as chlorophyll content, free amino acid, total protein content and total phenol increased with higher amounts of eggshells applied in cowpea (*Phaseolus vulgaris*). Studies have shown that calcium supports chlorophyll content indirectly by contributing to structural integrity, enzymatic function, and membrane stability, all of which are crucial for chlorophyll production and maintenance (Guo et al., 2023). Calcium is also an essential co-factor for several enzymes involved in the metabolism of amino acids. For instance, enzymes like *glutamate dehydrogenase* (important for amino acid synthesis) require calcium to function properly (Plaitakis et al., 2017). Adequate calcium levels can enhance the activity of these enzymes, leading to increased synthesis of amino acids, which are building blocks for proteins (Hildebrandt et al., 2015). Calcium is also involved in protein synthesis by regulating the function of ribosomes and other components of the protein synthesis machinery in the cell. It helps stabilize the structure of ribosomal subunits and influences the translation process (Schwarz & Blower, 2016). Therefore, it can be postulated that as calcium is increased, this will help plants improve on structural and enzymatic function, increasing chlorophyll content, producing enzymes important for amino acids formation and thus increasing proteins and leaf size as well. It can be deduced that the application of eggshells may be able to improve the vegetative growth by enhancing the height, number and width of the leaves of vegetables particularly *Brassica juncea*.

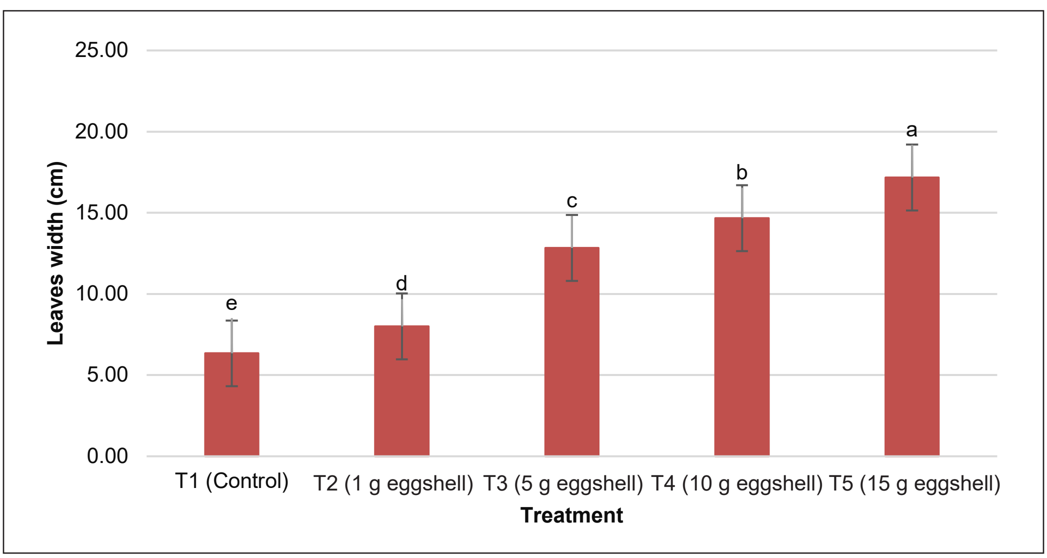

Figure 3. Mean width of leaves in *Brassica juncea* at harvest (56 DAS). Different letters in a column is significantly different according to Tukey's test ($p \leq 0.05$)

Soil pH

Significant difference in soil pH was observed in eggshell treatments as compared to control as displayed in Figure 4. The lowest soil pH was recorded at pH 5.5 for T1 whereas T5 recorded pH of 7.52 which was the highest soil pH compared to other treatments. Such increment up to 36% from the initial pH of 5.5 might be attributed to the enrichment of soil with calcium carbonate and others mineral and nutrients presence in eggshell since eggshells contain considerable amount of calcium carbonate ($CaCO_3$) which can act as an alternative to lime attributed to their high calcium content which able to neutralize the pH of acidic soils (King'Ori, 2011; Wang et al., 2023). It is well-established fact that soil pH of 6.5-7.5 is one of the good characteristics of a fertile soil having optimum physical, chemical and biological properties where all types of crops can be grown. Hence, the use of eggshell was useful in increasing soil pH from an acidic pH to neutral pH.

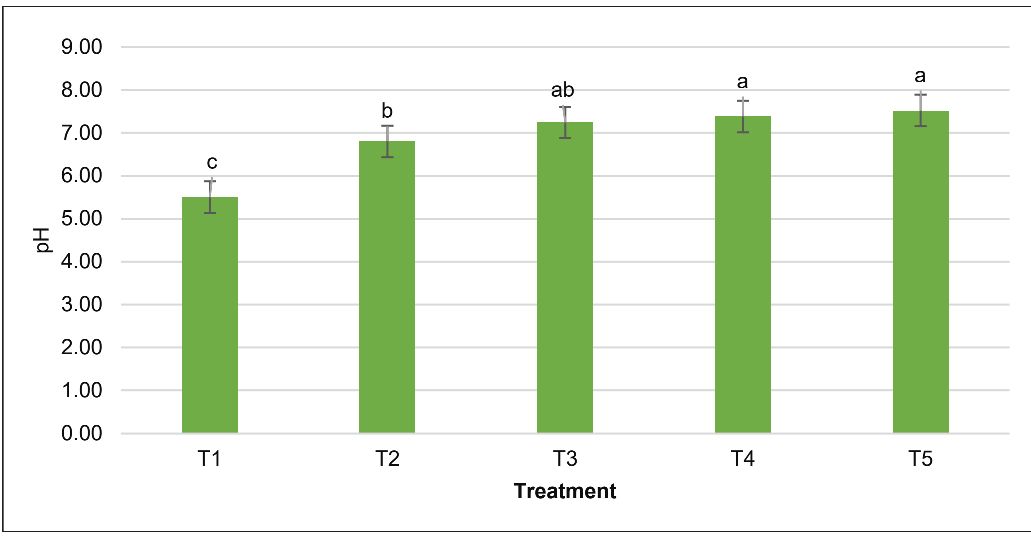

Figure 4. Mean soil pH planted with *Brassica juncea* at harvest (56 DAS). Different letters in a column is significantly different according to Tukey's test ($p \leq 0.05$) for treatments T1 (control), T2 (1 g eggshell), T3 (5 g eggshell), T4 (10 g eggshell), T5 (15 g eggshell)

Soil Nutrient Analysis

The means for soil nutrient analysis particularly phosphorus, potassium, calcium and magnesium are shown in Table 2. It was found that the calcium content was significantly the highest nutrient content, followed by phosphorus, potassium while the least was magnesium. The highest calcium content available in the soil was 3,429 mg kg^{-1} found in T5 at 15 g of eggshell application. This is to show that the application of eggshells might have contributed to the increase in available calcium in the soil. Such finding is

Table 2
Soil nutrient (P, K, Ca and Mg) at harvest (56 DAS)

Treatment	Soil available nutrient (mg kg^{-1})			
	P	K	Ca	Mg
T1(control)	814_{a}	723.9_{a}	1955_{c}	432.3_{b}
T2 (1 g eggshell)	711.43_{b}	517.2_{b}	2194_{b}	391.4_{c}
T3 (5 g eggshell)	559.4_{cd}	422.67_{c}	1875_{c}	297.27_{d}
T4 (10 g eggshell)	600.63_{c}	341.3_{d}	1585_{d}	251.23_{d}
T5 (15 g eggshell)	746.67_{b}	550.27_{b}	3429_{a}	550_{a}

Note. Different subscript within a particular nutrient (column) indicate significant difference between treatments

consistent with the report by Vu et al. (2022) whereby application of eggshells onto the soil affected pH and Ca content of the soil, where higher soil Ca was observed in higher eggshell powder rates. It can be deduced that eggshells can be a good alternative source for liming in alleviating soil acidity.

Plant Nutrient Analysis

Table 3 exhibit the mean leaf tissue nutrient analysis between treatments at harvest (56 DAS). Interestingly, only K content in *Brassica juncea* was found to be significantly different between treatments while no significant differences were found in P, Ca and Mg content in leaf tissue despite the additional increase of calcium content in the soil due to the application of the eggshells. It was also found that K is the highest nutrient content (3.3-4.0%) absorbed by the plant compared to the other nutrient (0.2-1.6%) where T1 recorded the highest K content in plant tissue (4.084 %) compared to other treatments (3.3-3.5 %). Studies done by Reeza et al. (2023) justified that plants will absorb nutrients that are needed for their growth and not according to the abundance of that particular nutrient in the soil. As such, several reports found that potassium is being highly absorbed by plants compared to other

Table 3
Leaf nutrient content (P, K, Ca and Mg) at harvest (56 DAS)

Treatment	Soil available nutrient (%)			
	P	K	Ca	Mg
T1	0.83_{ns}	4.084_{a}	1.468_{ns}	0.394_{ns}
T2	0.8372_{ns}	3.401_{b}	1.527_{ns}	0.380_{ns}
T3	0.756_{ns}	3.405_{b}	1.603_{ns}	0.355_{ns}
T4	0.6462_{ns}	3.521_{b}	1.469_{ns}	0.341_{ns}
T5	0.6016_{ns}	3.320_{b}	1.152_{ns}	0.270_{ns}

Note. Different subscript within a particular nutrient (column) indicate significant difference between treatments.
ns= non-significant

nutrients (Han et al., 2016; Reeza et al., 2023; Vimala et al., 2010). In contrast, magnesium was found to be the lowest nutrient taken up by the plant regardless of the amounts of treatments applied, a similar finding in the aforementioned soil nutrient analysis. Overall, it can be deduced that the application of eggshells had no significant effect on plant nutrient content in *Brassica juncea* except K content.

Absorption of Calcium from Eggshells into Plant Tissue

The form of Ca in eggshell is in $CaCO_3$ which is not readily-available for plant uptake whereby plants absorb Ca^{2+} from the soil solution since mass flow and root interception are the principal mechanisms of Ca transport to root surface. According to Ertürk (2020), the $CaCO_3$ in eggshell needs to be decomposed and converted to Ca which is available prior to plant uptake. This implies that the form of Ca is crucial for plant nutrient uptake. By utilizing crushed eggshells, the calcium content in the soil can be enhanced along with other nutrients found in the eggshell (Faridi & Arabhosseini, 2018; Silveira et al., 2016; Taylor & Locascio, 2004;). Moreover, the benefits of using crushed eggshells is that it requires little energy for preparation, but the drawback is that it requires time for eggshell degradation and decomposition in order to provide nutrients to the plant (Mitchell, 2005; Rai et al., 2014). However, this can be alleviated by introducing liquid and foliar form or tea fertilizer.

CONCLUSION

The study confirmed that different amounts of eggshells application can affect the growth of *Brassica juncea* (green mustard) by improving plant height, number of leaves and leaves width. The application of eggshells also increased soil pH up to 36% from the initial soil pH and increased soil calcium content. However, it did not affect the majority nutrient content in the plant. Also, eggshell indeed contains macronutrient and micronutrient that are essential for plant growth. Hence, household waste such as eggshells should be used as an organic liming material and organic fertilizer to increase soil pH and the growth of crops. Further research is warranted to explore optimal timing and method of application eggshells to maximize its benefits in Brassica species.

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Optimization of Harumanis Mango Leaves Extract for Enhanced Pharmacognostic Profile Using Response Surface Methodology Approaches

Ryan Vitthaya Peron[1], Amirul Ridzuan Abu Bakar[1]*, Mohd Asraf Mohd Zainudin[1], Ahmad Mukhlis Abdul Rahman[1], Nik Muhammad Azhar Nik Daud[1], Mohd Sharizan Md Sarip[1] and Nazar Mohd Zabadi Mohd Azahar[2]

[1]Faculty of Chemical Engineering and Technology, Universiti Malaysia Perlis, 02600 Arau, Perlis, Malaysia
[2]Faculty of Health Science, University Teknologi MARA, Cawangan Pulau Pinang, Kampus Bertam, 13200 Kepala Batas, Penang, Malaysia

ABSTRACT

Harumanis mango (*Mangifera indica* var Harumanis) is renowned for its fruit; however, its leaves remain an underexplored resource for therapeutic potential. Although other mango varieties have been studied for their medicinal properties, limited research has focused on the bioactive profile of Harumanis mango leaves, creating a critical knowledge gap. Hence, this study aims to fill the gap by optimizing the extraction of bioactive compounds from Harumanis leaves using dynamic maceration with ethanol and methanol as solvents. Phytochemical screening identified the presence of polyphenols, which were further validated by FTIR spectroscopy. Quantitative analysis revealed high levels of flavonoid and phenolic contents in both ethanolic and methanolic extracts, comprising total phenolic contents of 60.58 ± 0.005 and 36.73 ± 0.003 µg/g, respectively, and total flavonoid contents of 58.73 ± 0.015 and 46.25 ± 0.003 µg/g, respectively. The antioxidant evaluation of the ethanolic extract showed IC_{50} values of 52.905 ± 1.12 µg/mL, while the methanolic extract showed 84.649 ± 0.87 µg/mL; additionally, antibacterial tests further supported their therapeutic potential. These findings highlight the promising potential of these extracts for the development of natural therapeutics. The high concentrations of polyphenols and their bioactivity underscore the medicinal value of Harumanis leaves, highlighting their potential for use in herbal medicine. Overall, this study

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E-mail addresses:
rvitthaya@gmail.com (Ryan Vitthaya Peron)
amirulridzuan@unimap.edu.my (Amirul Ridzuan Abu Bakar)
asraf@unimap.edu.my (Mohd Asraf Mohd Zainuddin)
mukhlis@unimap.edu.my (Ahmad Mukhlis Abdul Rahman)
nikazhar@unimap.edu.my (Nik Mohd Azhar Nik Daud)
sharizan@unimap.edu.my (Mohd Sharizan Md Sarip)
nazarzabadi@uitm.edu.my (Nazar Mohd Azahar Nazar Zabadi)
* Corresponding author

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fills a crucial research gap, showcasing the untapped potential of Harumanis leaves as a natural resource for alternative therapies and unlocking new possibilities for innovative applications in the industrial and pharmaceutical fields.

Keywords: Dynamic maceration, Harumanis leaves, pharmacognosy, phytochemicals, response surface methodology

INTRODUCTION

Pharmacognostic approaches focus on discovering and developing new medications from natural sources. This field involves identifying, isolating, and characterizing plant bioactive compounds to create new treatments or enhance the existing ones. The increasing global demand for plant-based bioactive compounds, particularly antioxidants and antibacterial agents, has underscored their growing importance in the pharmaceutical and nutraceutical industries. These compounds are valued for their natural origin, safety, and efficacy in combating oxidative stress and microbial infections, contributing significantly to health and disease prevention. This trend reflects a shift towards sustainable plant-derived alternatives to synthetic compounds (Narayanankutty et al., 2024). Public interest in natural medicines is driven by their affordability, perceived safety, and potential effectiveness compared with synthetic drugs (Ekor, 2014). Exploring these remedies could unveil novel therapeutic targets and foster innovative and holistic approaches to healthcare.

The therapeutic potential of *Mangifera indica* (mango tree) has garnered increasing attention owing to its abundant bioactive components, making it a promising candidate for drug development. Native to South Asia, this tropical fruit is renowned for its culinary appeal and its traditional medicinal uses (Thivagaran et al., 2023). In Malaysia's Perlis region, the Harumanis mango stands out for its exceptional sweetness and aroma, making it a prized fruit. Its high antioxidant content suggests potential benefits in lowering the risk of progressive health disorders (Liu et al., 2018). Although fruit garners much attention, the therapeutic potential of mango leaves, especially those from the Harumanis variety, remains largely unexplored. Research has primarily focused on the fruit, leaving a gap in our understanding of the bioactive compounds in the leaves. Traditionally, mango leaves have been used to treat ailments like diarrhea, toothaches, and diabetes (Ediriweera et al., 2017). Scientific evidence supports these uses, revealing antidiabetic, anticancer, and antioxidant properties (Ganogpichayagrai et al., 2017). However, studies have not specifically addressed the Harumanis variety, which may have distinct therapeutic effects compared with other mango types.

Moreover, the efficacy of natural products may differ depending on factors such as plant maturity, environmental conditions, and harvest methods (Tungmunnithum et al., 2020). The concentration and type of compounds a plant produces are influenced by its species, genotype, and growth conditions (Isah, 2019). To maximize the benefits of mango leaves, optimizing the extraction methods to capture their full phytochemical potential is essential.

In the global pursuit of sustainable resource utilization, the exploration of underutilized biomass and innovative extraction techniques has gained momentum (Azhar et al., 2021; Fatt et al., 2021; Herman et al., 2021; Rohim et al., 2021). These studies have provided valuable insights into the identification of bioactive compounds. However, most of this research has primarily focused on characterizing chemical compositions rather than optimizing the extraction processes. This study addresses the gap by characterizing the bioactive profile of Harumanis mango leaves and optimizing extraction conditions to enhance yield and bioactivity. This approach highlights the importance of refining the extraction techniques to maximize the potential of plant-based resources for pharmaceutical and nutraceutical applications.

Recent studies have reported that Harumanis mango leaves contain notable phenolic and flavonoid compounds (Rahman et al., 2024). These compounds are known for their ability to neutralize free radicals, reduce oxidative stress, and exhibit antimicrobial activity, making them highly valuable in alternative medicine to promote overall health. Hence, the primary objective of this research is to screen and elucidate the active phytochemicals in Harumanis mango leaves using an optimized dynamic maceration technique. By addressing current knowledge gaps and challenges, this study aimed to highlight the medicinal potential of these leaves and explore their application in developing new nutraceutical and pharmaceutical products. Understanding the unique properties of Harumanis mango leaves and determining their optimal extraction conditions is crucial for leveraging their health benefits and advancing their use as phytomedicines. Ultimately, this study seeks to provide valuable insights into the potential of Harumanis mango leaves as a source of novel herbal drugs.

MATERIALS AND METHOD

This study used various analytical methods to explore the therapeutic potential of Harumanis leaves (Figure 1). This study aimed to identify the key bioactive compounds contributing to their therapeutic effects by carefully collecting and examining these data. Different analytical tools have provided a deeper and more detailed understanding of the pharmacological properties of these leaves.

Leaves Sample Preparation

Approximately 1 kg of pruned Harumanis leaves (ID: BT-D-B9P16-MA128) were collected from the Agricultural Complex of Jabatan Pertanian Negeri Perlis located at Bukit Temiang, Perlis. Figure 2 shows the leaf preparation process used in this study.

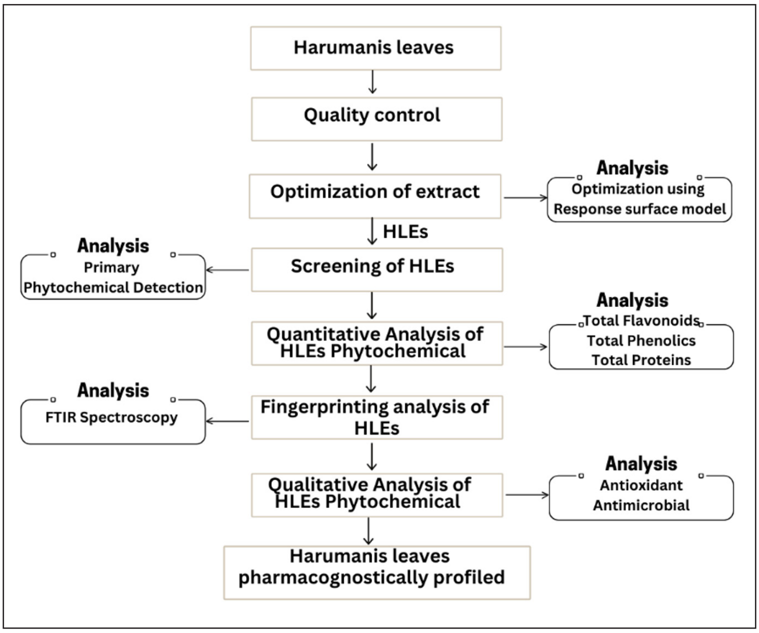

Figure 1. Research methodology overview

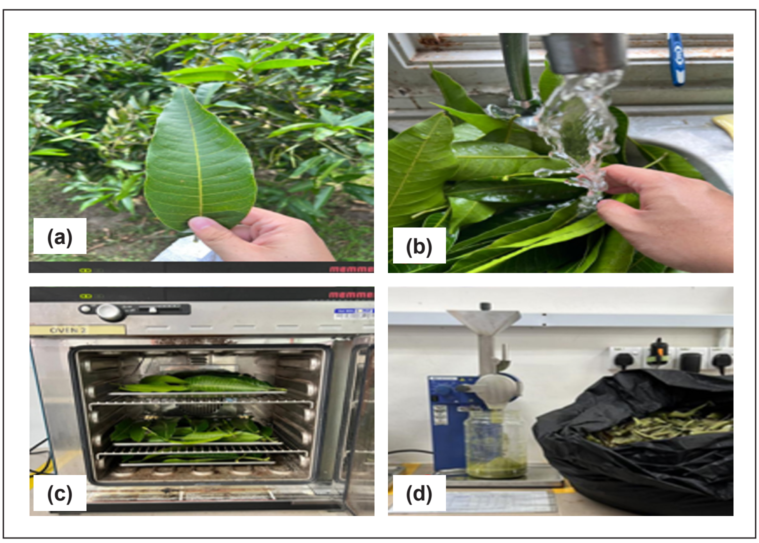

Figure 2. (a) Leaves collection; (b) washing and cleaning of leaves; (c) drying in oven; and (d) grinding

Foreign Particles Analysis

The leaves were initially weighed and spread to assess the presence of foreign particles in ground Harumanis leaves on a thin sheet. Any foreign material, such as small stones or insects, was identified and removed by visual inspection with a magnifying glass. Isolated foreign particles were weighed and divided according to the total sample weight. The result was multiplied by 100 to determine the percentage of foreign particles in the sample (Sani et al., 2018).

Total Ash Content Analysis and Acid Insoluble Ash Analysis

The total ash content was determined by weighing 2 g of Harumanis leaf powder and placing it into a silica crucible of 50 mL. The sample was initially heated using a hot plate, followed by combustion in a furnace at 400 °C for 4 h. The percentage of total ash was determined by dividing the ash mass by the original mass of the powder and multiplying it by 100, as described by Ngadiarti et al. (2022). The resulting ash in the crucible was mixed with 5 M hydrochloric acid (25 mL) for the acid-insoluble ash. The mixture was heated for 5 min. Then, the mixture was rinsed using 5 mL of hot water, leaving behind insoluble ash. The insoluble ash was then filtered through Whatman No. 1 filter paper and neutralized with hot water. The insoluble ash residue was returned to the crucible, dried, and incinerated until a constant weight was achieved. After cooling, the residue was measured, and the percentage of acid-insoluble ash was determined by dividing the weight of the residue by the initial weight of the sample powder and multiplying the result by 100, following the method described by Pradhan et al. (2010).

Total Moisture Content Analysis

The moisture content of powdered Harumanis leaves was assessed using an A&D Hybrid Moisture Analyzer. Approximately 2 g of each sample was weighed and loaded into the device to calculate the moisture content. To ensure reliable results, the procedure was repeated thrice.

Preparation of Harumanis Leaves Extracts (HLEs)

Design of Experiment

Response surface methodology (RSM) was applied to optimize dynamic maceration extraction. Hence, different extraction times, extraction temperatures, and solvent ratios were studied to analyse the results and characteristics of the extracted products. The experimental design employed in this study was the Box-Behnken design using Design Expert Version 7 software developed by State-Ease Inc. (Minneapolis, MN, USA). The parameters were selected based on the study of Ramlee et al. (2024) with slight modifications. The Box-Behnken design consisted of three components, each with three levels, resulting in 22 experiments. The study incorporated three distinct independent variables: time (A: 15–45 min), temperature (B: 30–60 °C), and solvent concentration or ratio (C: 0–100% in distilled water). The dependent variable was yielding percentage

(%). The design was implemented to optimize the dependent variables. The model was validated by comparing the experimental and predicted values (Rohilla & Mahanta, 2021).

Dynamic Maceration Preparation of HLEs

Approximately 5 g of powdered Harumanis leaf samples were measured and transferred into conical flasks. Subsequently, 50 mL of the solvent was introduced into each flask to aid the extraction process of the plant material. This methodology was replicated using different solvent concentrations, including distilled water, ethanol, a blend of ethanol and distilled water, methanol, and a blend of methanol and distilled water, each in a separate conical flask. The flasks were then subjected to controlled temperature conditions within a water bath to facilitate extraction through maceration, and the solution obtained after the extraction process was cooled and filtered using Whatman No. 1 filter paper. Next, the collected filtrate was placed in an oven and fully dried at 65 °C (Pereira et al., 2018). The extract yield was determined by dividing the weight of the crude extract by the weight of the powdered sample and multiplying the result by 100. This process was conducted thrice to enhance the accuracy.

Screening of HLEs

Preparation of Samples

A stock solution of approximately 10 mg/mL was prepared from the extract with the parameters that gives the highest yield. This stock solution was diluted with the appropriate solvent to achieve a final concentration of 10 mg/mL. The solution was filtered through Whatman No. 1 filter paper to remove any residues before proceeding with further tests.

Primary Phytochemical Detection

The phytochemicals in the HLEs were identified using standard qualitative methods, including hydrochloric acid, sodium hydroxide, and iron chloride. The presence of specific phytochemicals was indicated by the colour change that results from chemical reactions.

Flavonoids Detection

A few drops of a 10% sodium hydroxide solution were added to 2 mL of the aqueous extract. The addition of dilute hydrochloric acid caused the solution’s colour to change from yellow to colourless, confirming the presence of flavonoids (Hassali et al., 2022).

Phenols Detection

Ellargic’s test was performed to determine the presence of phenols. A few drops of 5% sodium nitrate ($NaNO_3$) and 5% gallic acid were added to the optimized ethanol and methanol HLEs. A brown precipitation indicated a positive result for phenols in the extracts (Jamil et al., 2023).

Saponins Detection

The presence of saponins was determined using foam tests. A 50 mg sample of HLEs was diluted with distilled water to a final volume of 20 mL and vigorously shaken for 2 min. The appearance of a 2 cm foam layer indicated the presence of saponins (Rai et al., 2023).

Protein Detection

The Biuret method was used to determine the presence of proteins. Approximately 1 mL of 4% NaOH solution and a few drops of 1% $CuSO_4$ were added to the optimized ethanol and methanol extracts of HLEs. A change in the colour of the extract solution to purple indicated a positive result in the protein test.

Quantitative Analysis of Phytochemical

Total Phenolic Content Analysis

Initially, a standard calibration curve using gallic acid was created at 31.25, 62.5, 125, 500, and 1000 ppm concentrations. For each concentration, 100 µL of gallic acid solution was mixed with 100 µL of 10% Folin-Ciocalteu reagent and 2 mL of 2% sodium carbonate (Na_2CO_3) solution. The mixture turned dark blue. After thorough mixing, the solutions were incubated for 30 min in the dark at room temperature. Absorbance was measured at 720 nm. This process was repeated thrice, and the calibration curve was plotted. The same procedure was followed to determine the total phenolic content in HLEs. The absorbance of the HLEs was measured and compared with the calibration curve. Total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of sample. The final calculation involved multiplying the concentration from the calibration curve by the extract volume and dividing it by the mass of the sample. All tests were conducted in triplicate (Nadzir et al., 2020).

Total Flavonoids Content Analysis

A calibration curve was constructed using quercetin at 31.25, 62.5, 125, 250, 500, and 1000 ppm concentrations. To determine flavonoid content, 250 µL of quercetin was mixed with 150 µL of 10% (w/v) aluminium chloride solution. After 5 min, 0.5 mL of 1 M sodium hydroxide and 575 µL of deionized water were added to the solution, resulting in a yellow solution. The absorbance was measured at 430 nm using a UV-Vis spectrophotometer. The total flavonoid content in the HLEs was determined by comparing their absorbance with the calibration curve, and the results were expressed as the concentration of flavonoids per unit mass of the extract (Bouyahya et al., 2018). All analyses were performed in triplicates.

Fingerprinting Analysis of HLEs

Fourier-Transform Infrared Spectroscopy (FTIR) using a Perkin Elmer instrument was employed for fingerprinting analysis of HLEs. Approximately 1 mL of 1 mg/mL HLE solution was analysed, with the FTIR spectrometer set to scan from 650 cm^{-1} to 4000 cm^{-1} at a resolution of 4 cm^{-1}. The resulting spectrum was examined to identify the key peak values and corresponding functional groups. The observed FTIR peaks were interpreted in relation to known bioactive compounds by correlating the identified functional groups with those typically found in specific bioactive molecules.

Qualitative Analysis of Harumanis Leaves

Determination of Antioxidant Activity

The antioxidant activity was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method, as described by Bakar et al. (2019). A calibration curve was established with ascorbic acid concentrations ranging from 31.25 to 2000 ppm. The DPPH solution was prepared by dissolving 3.2 mg of DPPH in 100 mL methanol. Then, 200 µL of ascorbic acid was added to 2.8 mL DPPH solution. The mixture was then shaken and incubated at room temperature for 1 h. Absorbance was measured at 517 nm using a UV/Vis spectrophotometer. DPPH radical scavenging activity (%) was calculated by subtracting the absorbance of the extract from that of the control, dividing by the control absorbance, and multiplying by 100. The IC_{50} values were determined from these results. All tests were performed in triplicates.

Determination of Antimicrobial Activity

The HLE's antimicrobial activities were conducted using a disc diffusion method against *Escherichia coli, Pseudomonas aeruginosa,* and *Bacillus cereus*. The agar plates were divided into four sections: two for the controls (2 mg/mL ciprofloxacin as a positive control and methanol as a negative control) and two for the extract solutions. The bacterial suspension was adjusted to a McFarland standard of 0.4. Sterile paper discs were impregnated with a 2 mg/mL extract solution and placed on agar plates. After 24 h of incubation at 35 °C, the inhibition zones were measured to assess antimicrobial activity.

RESULTS AND DISCUSSION

Quality Control Analysis

Quality control is critical to ensure the consistency, safety, and efficacy of herbal-derived drug preparations (Wang et al., 2023). The term "quality" refers to a drug's characteristics, including its identity, chemical and physical properties, purity, composition, and biological features. This analysis includes determining the total ash content, acid-insoluble ash, foreign matter, and total moisture content of Harumanis leaves as raw materials for developing herbal products. Animal-derived substances, including insects and microscopic microbial organisms, which may produce harmful toxins, are among the potential herbal contaminants (Kunle et al., 2012). Therefore, foreign matters, including molds, insects, spider webs, sands, and stones, were discarded from the leaves sample. In this study, the percentage of foreign matter in the Harumanis leaf sample was 2.28 ± 0.18%, which is considered low. This indicates that the percentage of foreign matter in the plant sample is relatively acceptable as it is less than 10% under pharmacopeial standards of herbal material. The total ash content was determined by measuring the total amount of material remaining after the burning. This includes "physiological ash", derived from the plant sample itself, and "non-physiological" ash, which is the residue of the extraneous matter adhering to the plant surface caused by environmental contamination. As the results obtained, the percentage of total ash content in plant raw materials was 9.02 ± 0.27%. This value is acceptable, as it is less than 14%, the maximum acceptable limit of total ash content recommended by the European Pharmacopoeia (Abdu et al., 2015).

Acid-insoluble ash is a component of total ash that indicates the presence of silica in the samples, most notably in sand and siliceous soil. Moreover, these values represent the concentrations of oxalates, carbonates, phosphates, oxides, and silicates (Shen et al., 2023). Therefore, these values are indicators of the quality of herbal remedies. According to the results, the percentage of acid-insoluble ash in the Harumanis leaves sample was 1.44 ± 0.25%, which is low and below 10%. This indicates the high purity of the herbal medicines. However, the presence of soil, for example, causes contamination that results in a high percentage of acid-insoluble ash that eventually disturbs the quality of the products.

To develop a high-quality herbal product, the percentage of total moisture content should be low because a higher moisture content attracts microbes to grow, which eventually contaminates the whole product. Therefore, it is important to control the moisture content of the raw material to ensure the product is of good quality. Based on the results, the total moisture content of the raw material was 8.93 ± 0.31%, which is considered low. This proves that Harumanis leaves have the potential to be developed into high-quality herbal products. Figure 3 illustrates the overall results of the quality control analysis of Harumanis leaves.

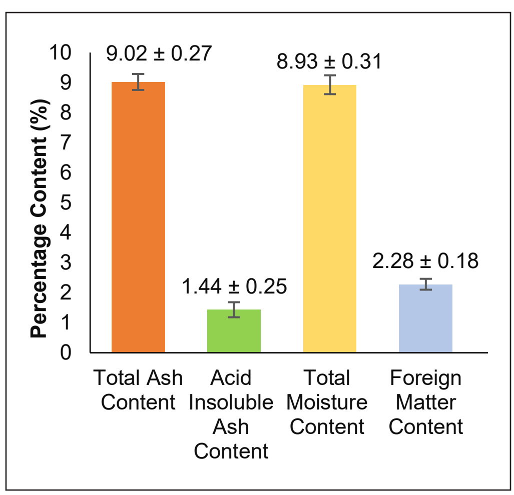

Figure 3. Percentage content for quality control analysis

Optimization of Dynamic Maceration Extraction

Dynamic maceration was optimized via RSM Box and–Behnken design to evaluate the best conditions for extracting bioactive compounds from Harumanis leaves. This approach was tested at various times, temperatures, and solvent ratios to determine the optimal extraction parameters. For ethanol extracts, the optimal conditions were 25 min of extraction time, 45 °C temperature, and a solvent ratio of 42% ethanol in 58% distilled water. This setup yielded a predicted extraction result of 22.13 ± 0.48%. In practice, the yield was slightly higher at 24.74 ± 0.32%. For extracts using methanol, the best conditions were an extraction time of 37 min, a temperature of 55 °C, and a solvent ratio of 44% methanol in 56% distilled water. This provided a predicted yield of 23.77 ± 0.26%, with an actual yield of 26.80 ± 0.13%. The interactions between different parameters and the extraction yield were recorded and are shown in Figure 4.

These optimized conditions (Table 1) were selected because they align with the goal of achieving an efficient and environmentally friendly extraction. The slightly higher actual yields compared to the predictions demonstrate that these conditions are effective and energy efficient. These results suggest that combining water with organic solvents, such as ethanol or methanol, enhances the extraction of soluble compounds, making it a better choice than using pure solvents alone. Similar findings have been reported in other studies, highlighting the effectiveness of aqueous methanol in plant extraction (Sultana et al., 2009).

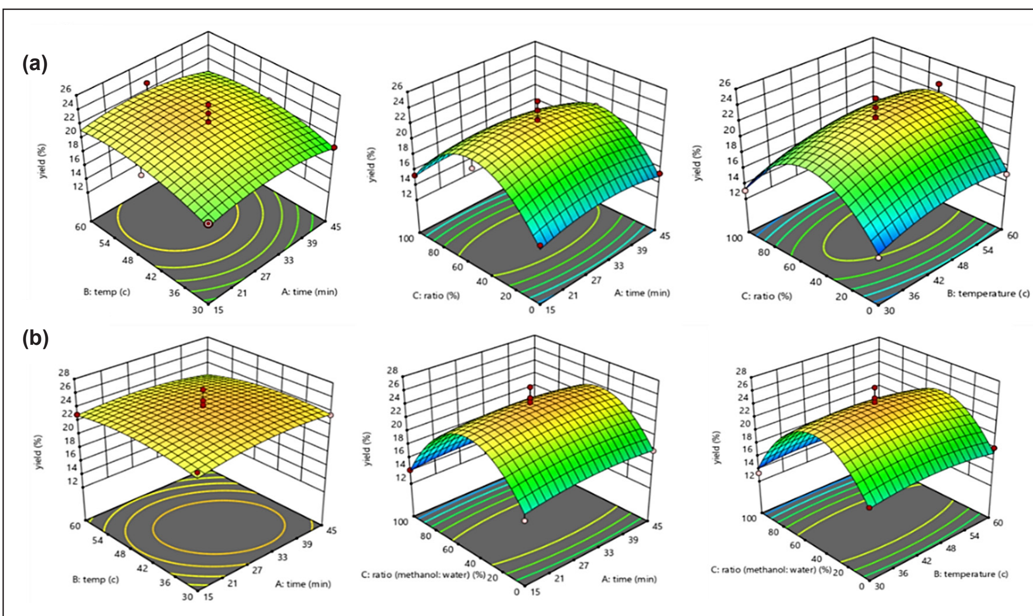

Figure 4. 3D contour for interactions of different parameters against yield of extract produced for extraction optimisation: (a) optimized ethanolic extraction conditions; and (b) optimized methanolic extraction condition

Table 1
Optimized parameters for dynamic maceration

Parameters	Ethanolic Leaves Extract	Methanolic Leaves Extract
Ratio (Solvent %)	42%	44%
Time (Min.)	25 Minutes	37 Minutes
Temperature (°C)	45 °C	55 °C
Yield (w/w %)	22.13 ± 0.48%	23.77 ± 0.26%

Primary Detection of Phytochemicals

Preliminary analysis of the phytochemical contents of HLEs was performed using four different types of tests (Biuret test, alkaline reagent test, Ellargic's test, and foaming test). Overall, these results suggest that HLEs contain a variety of phytochemicals, including flavonoids, proteins, phenols, and saponins, which may contribute to their therapeutic potential. Table 2 summarises the phytochemical tests, observations, and conclusions regarding the phytochemicals available in the optimized HLEs.

Table 2
Primary phytochemical screening for Harumanis leaves extracts (HLEs)

Test	Observation	Conclusion
Flavonoids Test	Yellow colour changed to colourless	Presence of flavonoids
Biuret Test	Purple hue formed	Presence of proteins
Ellargic's Test	Brown precipitation formed	Presence of phenols
Foaming Test (Saponins)	2 cm layer of foam formed above solution	Presence of saponins

Quantitative Control Analysis of Phytochemical

Total Phenolic and Flavonoids Contents of HLEs

The total phenolic content of HLEs was determined using the Folin-Ciocalteu method and gallic acid as the standard calibration (Figure 5).

Using UV-Vis spectroscopy, total phenolic content values were obtained from the standard calibration curve $y = 0.3262x + 0.0435$, where x is the absorbance and y is the phenolic concentration. The final concentration was measured in μg GAE/g in 5 mg extracts. According to the results obtained, the ethanolic extract has recorded the highest phenolic content with a value of 60.58 ± 0.005 μg/g, followed by methanolic extract (36.73 ± 0.003 μg/g). This study observed that the total phenolic content value was higher in ethanolic extracts than in methanolic extracts. This is because ethanol is a polar solvent with a strong capacity to dissolve a wide range of phenolic compounds (Jiménez-Moreno et al., 2019). The higher phenolic content in ethanolic extracts is attributed to the effectiveness of ethanol in solubilizing phenolic compounds, including flavonoids and antioxidants, from plant materials (Dai & Mumper, 2010). Therefore, the choice of solvent plays a critical role in the extraction process, and ethanol is particularly effective in extracting phenolic compounds, contributing to the higher phenolic content. Polyphenols are often soluble in organic solvents that are less polar than water (Mehmood et al., 2022). This indicates that an aqueous mixture containing polar solvents, such as ethanol, is favourable for phenolic compound extraction from Harumanis leaves. Thus, ethanol is a good solvent for extracting polyphenols from HLEs.

The total flavonoid content of the HLEs was determined by an aluminium chloride colorimetric test using quercetin as a standard calibration (Figure 6).

UV-Vis spectroscopy obtained TFC values from the calibration curve $y = 2.7169x - 0.1484$, where x is the absorbance and y is the flavonoid concentration. The final concentration was measured in µg QE/g in 5 mg extracts. According to the results obtained, ethanolic extracts possessed the highest flavonoid content with a value of 58.73 ± 0.015 µg/g, followed by methanolic extracts with 46.25 ± 0.003 µg/g. This means that the polarity of the solvent affects the TFC values, and the optimum value of TFC was obtained using ethanol as a solvent for extraction. This higher flavonoid content in ethanolic extracts is attributed to the effectiveness of ethanol in solubilizing flavonoids, a subgroup of phenolic compounds from plant materials. The polarity of ethanol allows it to extract a broader spectrum of flavonoids, contributing to the higher flavonoid content observed in ethanolic plant extracts than in methanolic extracts (Ekin et al., 2017). Ethanol proved to be the most reliable solvent for extracting flavonoid content. This is because ethanol is more advantageous than water and

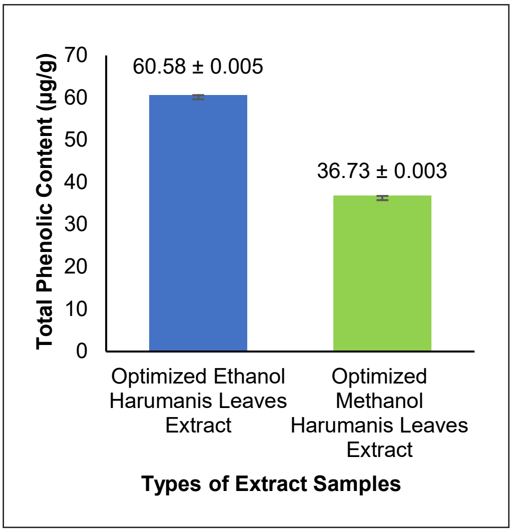

Figure 5. Total phenolic content of optimized ethanolic and methanolic Harumanis leaves extracts (HLEs)

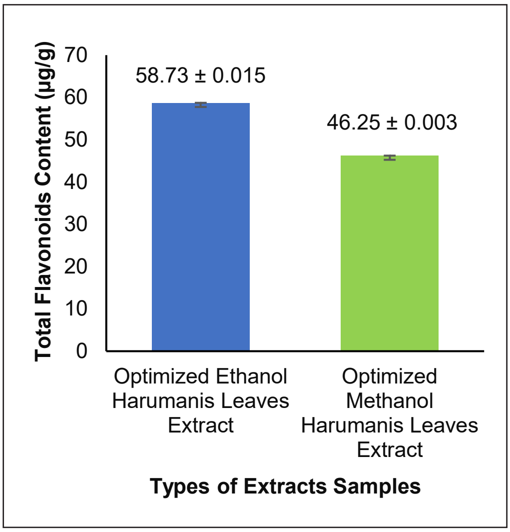

Figure 6. Total flavonoids content of ethanolic leaves and methanolic Harumanis leaves extracts (HLEs)

methanol regarding traceability (Abdu et al., 2015; Dai & Mumper, 2010). A study reported that maceration of mango leaves with a 1:10 (w/v) ethanol ratio also produced significant results in the expression of flavonoids (Sari et al., 2022). Hence, it can be concluded that an organic solvent, such as ethanol, was particularly effective in extracting the flavonoid content of Harumanis leaves.

Fourier Transform Infrared Spectrometry (FTIR) Analysis of HLEs

Figure 7 shows the FTIR analysis of the ethanolic and methanolic extracts of HLEs, highlighting the extracts' functional groups and chemical composition.

The FTIR spectrum of ethanolic HLE revealed a complex chemical composition indicative of the diverse natural products present. The broad absorption band at 3384.51 cm^{-1} corresponds to O-H stretching vibrations, suggesting the presence of hydroxyl

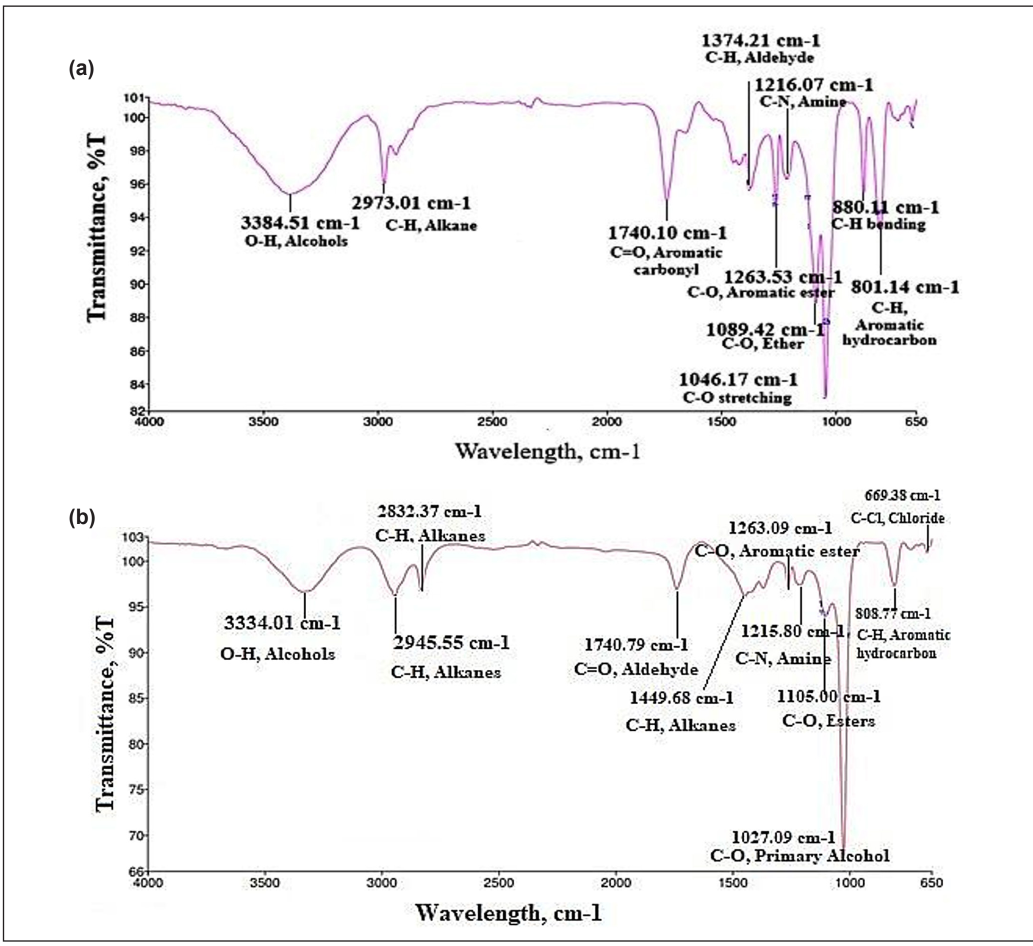

Figure 7. Fourier-Transform Infrared Spectroscopy (FTIR) spectra of Harumanis leaves extracts (HLEs): (a) ethanolic HLE; and (b) methanolic HLE

groups typically found in alcohols, a common feature in many plant-derived compounds. The peak at 2973.01 cm^{-1}, associated with C-H stretching, points to alkyl groups, which are prevalent in various organic molecules. The strong band at 1740.10 cm^{-1}, characteristic of C=O stretching in aromatic carbonyl compounds, indicates the presence of carbonyl functionalities, likely from aldehydes or ketones. Further, the band at 1374.21 cm^{-1} suggests aldehydic C-H bending, reinforcing the presence of aldehyde groups. The peaks at 1263.53 and 1216.07 cm^{-1}, corresponding to C-O and C-N stretching, respectively, imply the presence of aromatic esters and amine groups, typical of complex plant secondary metabolites. Additionally, the absorption bands at 1098.42 and 1046.17 cm^{-1} indicate C-O stretching, suggesting ether linkages within the extract. Lastly, the bands at 880.11 and 801.14 cm^{-1} confirmed the presence of aromatic hydrocarbons through out-of-plane C-H bending, highlighting the presence of aromatic rings.

Next, the FTIR spectrum of the methanolic HLE displayed several prominent peaks that corresponded to functional groups commonly associated with various phytochemicals. A broad band observed around 3334 cm^{-1} indicates the presence of O-H stretching vibrations characteristic of alcohols and phenolic compounds. The peaks at 2945 and 2832 cm^{-1} are associated with the C-H stretching vibrations of alkanes, suggesting the presence of long-chain hydrocarbons. The band at 1740 cm^{-1} corresponds to aldehydes' carbonyl (C=O) stretching, while the peak at 1449 cm^{-1} suggests C-H bending, further confirming the presence of alkanes. The sharp peak at 1263 cm^{-1} can be attributed to the C-O stretching of aromatic esters, indicating esterified compounds in the leaf extract. The C-N stretching was observed at 1215 cm^{-1}, suggesting the presence of amines, which may be linked to alkaloids or amino acids. Additionally, the peak at 1027 cm^{-1} corresponds to the C-O stretching of primary alcohols, while the band at 1105 cm^{-1} represents ester C-O stretching, suggesting the presence of various esters. The smaller peaks around 669 and 808 cm^{-1} indicate the presence of chlorides (C-Cl stretching) and aromatic hydrocarbons, respectively. Together, these spectra demonstrated the diverse chemical composition of HLEs, featuring structures of alcohols, esters, aldehydes, alkanes, and aromatic compounds, which are characteristic of phenolic and flavonoid compounds, contributing to their bioactivity and underscoring their potential therapeutic benefits.

Antioxidant Assay

Table 3 presents the DPPH scavenging activities of the HLEs. The study revealed that the ethanolic extract exhibited remarkable antioxidant activity, boasting the lowest IC_{50} value of 52.905 ± 1.12 μg/mL. In comparison, methanolic leaves showed a slightly higher IC_{50} value of 84.649 ± 0.87 μg/mL. A lower IC_{50} value indicates that a smaller concentration is required for 50% inhibition, highlighting the potent antioxidant capability of the ethanolic extract (Hassanpour & Doroudi, 2023). As a polar solvent, ethanol effectively extracts a

Table 3
DPPH scavenging activity of Harumanis leaves extracts (HLEs)

Extracts	DPPH Scavenging Activity Value (IC_{50})
Ascorbic acid (standard calibration)	0.7029 ± 0.97 μg/mL
Optimized ethanolic HLE	52.905 ± 1.12 μg/mL
Optimized methanolic HLE	84.649 ± 0.87 μg/mL

wide range of phenolic compounds, including flavonoids and antioxidants, from mango leaves. These bioactive compounds are known for their ability to scavenge free radicals, such as DPPH, which is crucial for combating oxidative stress.

The ethanolic extract has a higher phenolic content, enhanced DPPH radical scavenging activity, and superior antioxidant effects than the methanolic extract (Baliyan et al., 2022; Gondi & Rao, 2015; Kingne et al., 2019). This efficacy is attributed to the ability of ethanol as a polar solvent to extract a richer spectrum of antioxidant agents, including potent phenolic acids (Koirala et al., 2024). In contrast, the lower radical-scavenging activity of the methanolic extract reflects the reduced concentration of these valuable antioxidants. This study underscores ethanol as the optimal solvent for maximizing the extraction of antioxidant compounds from HLEs, with promising enhanced therapeutic benefits against oxidative stress.

Anti-Bacterial Assay

The antibacterial activity of the HLEs was assessed using the disc diffusion method against three diverse bacteria: *Escherichia coli, Pseudomonas aeruginosa*, and *Bacillus cereus*. This method visualizes the inhibitory zones around filter paper discs after incubation, reflecting the antibacterial effects of the extracts. The diameters of these zones directly correlate with the potency of the extracts against bacteria. To benchmark their efficacy, the samples were compared against ciprofloxacin, a broad-spectrum antibiotic, glycerin (glycerol), known to enhance plant extracts' antimicrobial activity, and Dettol, a renowned hand sanitizer. This comparative study aimed to ascertain the effectiveness of HLEs in inhibiting microbial growth, highlighting their potential as natural antibacterial agents. Based on Table 4, it can be seen that the HLEs in different extracts possess antimicrobial activity against all three tested microbes. Ethanolic extracts, especially ethanol with distilled water, exhibited the largest inhibition diameter against *E. coli* (31 mm) and consistent inhibition against *P. aeruginosa* (13 mm) and *B. cereus* (18 mm). Whereby its counterpart of ethanolic leaves in glycerin exhibits an inhibition diameter of 29 mm against *B. cereus*, 27 mm against *P. aeruginosa* and 13 mm against *E. coli*. Antibacterial activity is categorized based on the diameter of the inhibition zone: less than 5 mm is considered weak, 5–9 mm is moderate, 10–19 mm is strong, and over 20 mm is extremely strong (Gulcin & Alwasel, 2023). This classification reflects the antibacterial efficacy of the ethanolic leaf extracts against various microorganisms. This indicates that the antibacterial properties of ethanolic leaves against *E. coli* are powerful when mixed with distilled water. When combined with glycerin, the growth inhibition of *P. aeruginosa* and *B. cereus* was very strong. This also shows that ethanolic leaves have the highest antimicrobial properties.

This finding also correlates with previous results where the highest antimicrobial properties were observed in ethanolic leaves, as the extract showed the highest antioxidant activity based on the lowest IC_{50} value. In the microbial environment, the higher content of phenols and flavonoids interacts with microbial cells at a molecular level, disrupting cellular processes and structures (Huang et al., 2022). They can penetrate microbial cell walls, leading to cell lysis and death. Furthermore, these compounds can interfere with microbial communication systems, affecting microbial colonization and survival. The antimicrobial activity of phenols and flavonoids in the environment is crucial for combating microbial infections and controlling their populations (Omidfar et al., 2023). However, methanolic leaves in glycerin also demonstrated promising results, as they strongly inhibited the growth of *P. aeruginosa* (30 mm) and *B. cereus* (27 mm). Both ethanolic and methanolic leaf

Table 4
Summary of antimicrobial tests for Harumanis leaves extracts (HLEs)

Test organism	Sample	Diameter of inhibition zone (mm)
E. coli	Ciprofloxacin	23
	Glycerin	18
	Dettol Hand Sanitizer	9
	Ethanolic Leaves + Glycerin	13
	Ethanolic Leaves + Distilled Water	31
	Methanolic Leaves + Glycerin	16
	Methanolic Leaves + Distilled Water	19
P. aeruginosa	Ciprofloxacin	33
	Glycerin	31
	Dettol Hand Sanitizer	9
	Ethanolic Leaves + Glycerin	27
	Ethanolic Leaves + Distilled Water	13
	Methanolic Leaves + Glycerin	30
	Methanolic Leaves + Distilled Water	21
B. cereus	Ciprofloxacin	35
	Glycerin	32
	Dettol Hand Sanitizer	6
	Ethanolic Leaves + Glycerin	29
	Ethanolic Leaves + Distilled Water	18
	Methanolic Leaves + Glycerin	27
	Methanolic Leaves + Distilled Water	19

extracts were better antibacterial agents than Dettol hand sanitizer. This may be because the product is commonly used for sanitization to lower concentrations of microbes on the skin's surface. Thus, the product exhibited mediocre antibacterial properties when tested via the disc diffusion method (Ecevit et al., 2022). Overall, the results showed that both HLEs had potent antimicrobial properties, whereas ethanolic leaves showed better antimicrobial properties, as supported by previous tests. Therefore, these results support using HLEs as potential medicinal herbal products.

Collectively, HLEs hold potential as natural alternatives to synthetic antioxidants and antibacterial agents for pharmaceutical applications. These extracts, along with compounds such as phenolic acids and flavonoids, exhibit potent antioxidant and antibacterial properties, effectively neutralize free radicals, and inhibit bacterial growth. Notably, HLEs have demonstrated robust free radical scavenging activity, consistent with similar findings for other mango varieties, including Tommy Atkins, Alphonso, and Kent (Sousa et al., 2023). With increasing consumer demand for plant-based and eco-friendly products, HLEs offer a viable alternative to synthetic compounds in pharmaceutical formulations, addressing therapeutic needs while minimizing environmental impacts.

CONCLUSION

This study highlights the therapeutic potential of Harumanis mango leaves, which contain a rich polyphenol composition. The leaves were confirmed to be of high quality and exhibited promising medicinal properties. Ethanolic HLEs stood out, showing the highest levels of total phenolic and flavonoid contents, positioning them as strong candidates for therapeutic use. FTIR analysis further identified various phytochemicals, such as phenols, alkanes, alkenes, and alcohols, contributing to the antioxidant capabilities of the extracts.

The superior antioxidant and antibacterial properties of ethanolic HLEs make it a promising candidate for natural therapeutic development. Their demonstrated efficacy highlights their potential applications in managing oxidative stress-related conditions and microbial infections, providing a sustainable and eco-friendly alternative to synthetic agents. HLEs offer the potential to develop innovative herbal formulations that promote human health while meeting the growing demand for environmentally sustainable solutions. Their commercialization could also drive economic growth by enabling the production of high-demand, sustainable products. Advancing research in this area is essential to bridge the gap between laboratory studies and practical applications, thus paving the way for the widespread adoption of HLEs as effective therapeutic agents. By supporting the development of eco-friendly alternatives to synthetic compounds, the commercialization of HLEs contributes to environmental sustainability and provides substantial economic benefits. These efforts will firmly establish HLEs as a valuable resource in natural therapeutics, advancing health outcomes and sustainability.

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Mitigating Iron Toxicity and Enhancing Rice Growth Through Periodic Flooding in Tropical Ultisol Fields with Low Productivity

Sri Utami Lestari[1,2,4], Dyah Roeswitawati[1], Syafrani Syafrani[2], Dian Indratmi[1], Syarif Hasibuan[2] and Indra Purnama[2,3,4]*

[1]*Department of Agrotechnology, Faculty of Agriculture, Universitas Muhammadiyah Malang, Malang 65144, East Java, Indonesia*

[2]*Department of Agrotechnology, Faculty of Agriculture, Universitas Lancang Kuning, Pekanbaru 28261, Riau, Indonesia*

[3]*Graduate School of Agricultural Sciences, Universitas Lancang Kuning, Pekanbaru 28261, Riau, Indonesia*

[4]*Center for Sustainable Tropical Agricultural Research, Universitas Lancang Kuning, Pekanbaru 28261, Riau, Indonesia*

ABSTRACT

Iron toxicity is a major constraint in rice cultivation on tropical Ultisol soils, particularly in fields with a history of low productivity. Continuous flooding in paddy systems exacerbates this issue by increasing Fe^{2+} availability, leading to toxicity at high concentrations. This study evaluated periodic flooding and drying as a sustainable water management strategy to mitigate Fe^{2+} toxicity and enhance rice growth. A greenhouse experiment was conducted using a completely randomized design with five treatments and five replications, assessing different flooding and drying intervals. Soil pH and Fe^{2+} concentrations were measured at the end of the experiment, along with key rice growth and yield parameters. Results showed that extended drying intervals significantly improved soil pH and reduced Fe^{2+} concentrations, mitigating toxicity. The treatment with three weeks of drying followed by three weeks of flooding resulted in the lowest Fe^{2+} concentration (4.08 ppm) and the most stable pH conditions. However, the highest rice productivity was observed in the two-week flooding–two-week drying treatment, which promoted increased plant height, tiller number, and grain yield. These findings highlight the potential of periodic flooding as a practical and sustainable approach to improve soil health and rice productivity in iron-toxic Ultisol fields. This study contributes to developing effective water management strategies for rice cultivation in

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E-mail addresses:
sriutami@unilak.ac.id (Sri Utami Lestari)
dyahwati@umm.ac.id (Dyah Roeswitawati)
syafrani@unilak.ac.id (Syafrani Syafrani)
dian@umm.ac.id (Dian Indratmi)
syarifhasibuanm@gmail.com (Syarif Hasibuan)
indra.purnama@unilak.ac.id (Indra Purnama)
* Corresponding author

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marginal tropical soils, addressing both environmental sustainability and agricultural productivity. Future research should explore the long-term impacts of periodic flooding on soil nutrient dynamics and its applicability across diverse tropical agroecosystems.

Keywords: Periodic flooding, soil iron management, sustainable water practices, tropical soil health, ultisol paddy fields

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INTRODUCTION

Rice (*Oryza sativa L.*) is a staple crop for over half of the global population, particularly in tropical regions, where it serves as a critical source of calories and livelihood for millions of smallholder farmers (Purnama et al., 2023a). However, rice cultivation in marginal tropical soils, such as Ultisols, faces numerous challenges due to inherent soil limitations (Saleem et al., 2023). Ultisols, which dominate large areas in Southeast Asia, including Indonesia, are characterized by their acidic nature, low nutrient availability, and high concentrations of exchangeable aluminium and iron (Mureithi et al., 2024; Soelaeman & Haryati, 2012). These conditions severely constrain crop productivity, necessitating innovative soil and water management strategies to unlock the agricultural potential of such soils.

Iron (Fe) toxicity is a particularly significant constraint in rice cultivation on Ultisols, especially under continuous flooding conditions. Waterlogged soils promote the reduction of Fe^{3+} to Fe^{2+}, a highly soluble and toxic form of iron that interferes with root development, nutrient uptake, and photosynthesis (Aung & Masuda, 2020; Leinonen, 2023; Ponnamperuma, 1972; Wairich et al., 2024). Over time, high Fe^{2+} concentrations can lead to severe yield losses, as observed in many newly opened Ultisol fields where farmers struggle with persistently low productivity despite the use of organic and synthetic fertilizers (Andrew et al., 2020; Purnama et al., 2023b). These soils often exhibit unstable chemical properties, with highly acidic pH levels and poor nutrient cycling, further exacerbating the challenges of rice cultivation.

Although previous studies have demonstrated the potential of periodic flooding and drying (intermittent irrigation) to mitigate Fe toxicity by promoting soil redox dynamics, much of this research has been conducted in established rice systems with relatively stable soil properties (Andrew et al., 2020; Dossou-Yovo et al., 2023; Saidi et al., 2021). For example, Andrew et al. (2020) and Dossou-Yovo et al. (2023) showed that alternating wet and dry conditions reduce Fe^{2+} concentrations and stabilize soil pH, thereby minimizing iron toxicity and supporting rice productivity. However, in established paddy fields, long-term cultivation fosters the accumulation of soil organic matter and the development of adapted microbial communities, which actively regulate Fe^{2+} solubility and buffer redox fluctuations (Kögel-Knabner et al., 2010; Ninin et al., 2024; Tan et al., 2019). The presence of Fe–organic matter complexes and a well-developed microbial iron-reducing community helps stabilize Fe^{2+} levels under flooded conditions while preventing excessive oxidation when drained, making the response to intermittent irrigation more predictable (Pett-Ridge & Firestone, 2005; Wu et al., 2019). These studies primarily focus on systems with mature nutrient cycling and consistent management practices, whereas newly opened Ultisols exhibit more pronounced fluctuations in redox potential due to their undeveloped biological and chemical equilibria.

Newly opened Ultisol fields, by contrast, are characterized by high acidity ($pH < 5$), unstable redox conditions, and excessive soluble Fe^{2+} concentrations, which are not yet fully equilibrated due to the lack of long-term cultivation. Unlike in well-established paddy soils, where Fe^{2+} accumulation is moderated by organic matter interactions and microbial Fe reduction-oxidation cycles, newly opened Ultisols lack these buffering mechanisms, leading to rapid and unpredictable changes in Fe^{2+} solubility (Pett-Ridge & Firestone, 2005; Wu et al., 2019). Studies have shown that tropical soils recently converted to paddy systems experience erratic Fe^{2+} mobilization due to sharp redox fluctuations, which only stabilize after several years of cultivation (Wu et al., 2019). Similarly, in newly cultivated Ultisols, the absence of well-established microbial and chemical equilibria leads to higher variability in Fe^{2+} solubility compared to older paddies, where organic matter accumulation and iron oxide transformations help buffer redox cycles (Ninin et al., 2024).

While the effectiveness of periodic flooding has been established in traditional paddy systems, its impact on Fe dynamics, pH stabilization, and rice productivity in newly opened Ultisol fields remains underexplored. The extreme conditions in Ultisols, including rapid changes in redox potential and nutrient availability, pose challenges for extrapolating findings from established systems to these marginal tropical soils. This difference raises questions about whether the same periodic flooding strategies used in mature paddy fields will yield similar benefits in newly cultivated Ultisols, where chemical instability could either amplify or diminish the expected effects. This gap in understanding hinders the development of water management strategies tailored to newly cultivated Ultisols.

This study addresses the critical knowledge gap by examining the effects of periodic flooding on Fe concentrations in floodwater, soil pH, and rice growth and productivity in newly opened Ultisol fields. Unlike previous research, which often emphasizes broader soil health metrics and microbial activity (Freitas et al., 2024; Majumdar et al., 2023), this study focuses specifically on how varying flooding and drying intervals influence Fe^{2+} solubility, pH fluctuations, and observable plant responses such as growth and yield. Given the extreme redox variability and chemical instability in newly cultivated Ultisols, understanding how periodic flooding modulates Fe^{2+} dynamics in these soils is crucial for developing effective water management strategies. By targeting these specific parameters, the study provides actionable insights into mitigating Fe toxicity and improving rice productivity in newly cultivated Ultisols. These findings hold broader significance for enhancing food security in tropical regions where rice is a staple crop and where iron toxicity limits yields. Furthermore, optimizing periodic flooding as a water management strategy not only improves crop resilience but also promotes efficient water use, reducing the need for continuous submergence and mitigating risks associated with water scarcity and climate variability in rice-growing areas.

MATERIALS AND METHODS

Study Site and Soil Collection

The study was conducted in a greenhouse at Universitas Lancang Kuning, Riau, Indonesia. Ultisol soil was collected from paddy fields in Muara Kelantan Village, Siak Regency, Riau, Indonesia (0.577° N, 101.424° E). The fields have been cultivated for five years, utilizing both organic and synthetic fertilizers. Despite these efforts, persistent low productivity and symptoms of iron toxicity were observed. Soil samples were taken from the top 0–20 cm layer using a composite sampling method to represent the overall field characteristics. The collected soil was air-dried, sieved to 2 mm, and analysed for initial properties, including pH, organic carbon, nitrogen, phosphorus, and exchangeable iron (Fe). Soil analysis followed standard protocols as outlined by Association of Official Analytical Chemists (1995).

Experimental Design

The experiment was designed as a completely randomized design (CRD) with five treatments and five replications, resulting in a total of 25 experimental units. Each unit consisted of a polybag filled with 10 kg of air-dried Ultisol soil. The treatments evaluated different flooding and drying intervals to assess their effects on Fe dynamics, soil pH, and rice (*Oryza sativa* L.) productivity.

The treatments included continuous flooding throughout the experiment (A0), alternating 2 weeks of flooding followed by 2 weeks of drying (A1), alternating 2 weeks of drying followed by 2 weeks of flooding (A2), alternating 3 weeks of flooding followed by 3 weeks of drying (A3), and alternating 3 weeks of drying followed by 3 weeks of flooding (A4). Water management across treatments followed distinct flooding and drying cycles. During the flooding phases, water levels were maintained at 5 cm above the soil surface, as depicted in the schematic

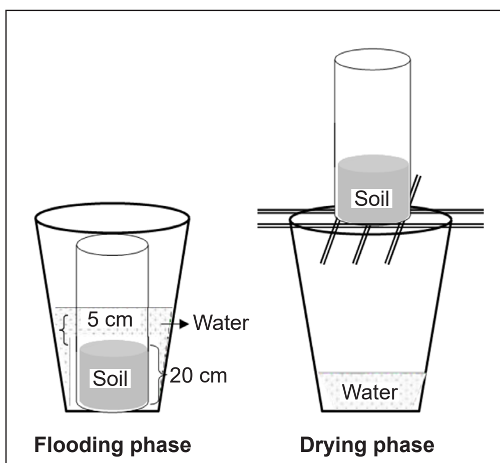

Figure 1. Schematic representation of the experimental flooding and drying treatments

diagram (Figure 1). During the drying phases, polybags were elevated to allow complete drainage, mimicking natural drying conditions in the field. The transition between flooding and drying was carefully controlled to ensure accurate simulation of alternating anaerobic and aerobic soil conditions.

Rice Cultivation

The rice variety *Inpari 42* was selected due to its adaptability to lowland conditions and moderate tolerance to Fe toxicity. Seeds were pre-germinated by soaking in water for 48 hours before being sown directly into the polybags. Three seedlings were transplanted into each polybag, and standard agronomic practices were followed. Fertilizers were applied in three stages: basal application at planting, during the tillering stage, and at panicle initiation. Urea was applied at 50 kg/ha, SP-36 at 75 kg/ha, and KCl at 50 kg/ha to meet the nitrogen, phosphorus, and potassium requirements of the crop. Pest and disease management was carried out as required, using environmentally friendly pesticides to ensure crop health (Purnama et al., 2025, 2024).

Data Collection and Measurements

Data collection focused on soil chemical parameters and plant growth and productivity. Soil pH was measured to evaluate the effects of periodic flooding and drying on the chemical properties of Ultisol soil. Measurements were conducted at specific intervals during the experiment to capture changes in pH associated with different water management regimes. A calibrated digital pH meter (Hanna Instruments, HI 98191) was used, following a 1:2 soil-to-water ratio protocol.

For the continuous flooding treatment (A0), pH was measured weekly to monitor soil acidity stability under anaerobic conditions. For periodic flooding and drying treatments (A1, A2, A3, A4), pH measurements were taken at the end of each flooding phase to capture the cumulative effect of prolonged reducing conditions on Fe^{2+} solubility. This timing was selected because Fe^{2+} progressively accumulates under anaerobic conditions and typically reaches peak concentrations at the end of the flooding cycle, making this phase the most critical for evaluating iron toxicity risks. While monitoring pH across both flooding and drying phases could provide additional insights into dynamic redox fluctuations, logistical constraints—such as field accessibility and sample preservation—limited the feasibility of continuous measurement. Thus, pH was recorded during the phase where Fe^{2+} levels were expected to be most representative of reducing conditions. Soil samples were collected from each polybag at a depth of 5–10 cm to ensure consistency across treatments. Each sample was thoroughly mixed before preparing a 1:2 soil-to-water suspension, which was stirred and allowed to settle for 30 minutes prior to pH measurement. All measurements were replicated three times per sample to ensure accuracy.

Fe^{2+} concentrations in the soil solution were analysed at the end of the study period, specifically after the final flooding phase for each treatment, using an Atomic Absorption Spectrophotometer (PerkinElmer AAnalyst 800), following the method described by Mahender et al. (2019). This approach was chosen to capture the cumulative effect of periodic flooding and drying cycles on Fe^{2+} solubility, providing a representative measurement of iron availability under the prevailing redox conditions at the conclusion of the experiment. Plant growth was assessed by measuring plant height and the number of tillers per plant at weekly intervals throughout the growth cycle. At harvest, yield components were recorded, including the number of panicles per plant, the percentage of filled grains, 100-grain weight, and dry grain weight per polybag, after drying to 12% moisture content.

Statistical Analysis

The collected data were analysed using Analysis of Variance (ANOVA) to evaluate the effects of treatments on soil and plant parameters. Duncan's Multiple Range Test (DMRT) was applied to compare treatment means when significant differences ($p < 0.05$) were identified. Statistical analyses were performed using SPSS software (version 25.0).

RESULTS AND DISCUSSION

Initial Soil Characteristics

The initial analysis of the soil from newly opened paddy fields in Muara Kelantan Village, Siak Regency, Indonesia revealed key chemical properties that characterize the challenges of cultivating rice in marginal Ultisol soils. Table 1 summarizes the key chemical properties of the soil, emphasizing the balance between nutrient potential and challenges for sustainable rice cultivation.

The chemical analysis showed that the soil exhibited a highly acidic pH of 4.70, which significantly limits nutrient availability and promotes the solubility of toxic elements such as aluminium (Al) and iron (Fe). Acidic soils are a common characteristic of Ultisols, particularly in tropical regions, and often require careful management to improve their suitability for agricultural purposes (Swe & Funakawa. 2023). Organic carbon content was very high at 14.3%, reflecting significant accumulation from previous organic matter applications. Despite this, the total nitrogen content was moderate at 0.43%, resulting in a very high carbon-to-nitrogen (C/N) ratio of 33.3. This imbalance suggests slow organic matter decomposition and limited nitrogen availability for plant uptake, which can constrain microbial activity and nutrient cycling in the soil (Kuśmierz et al., 2023; Li et al., 2022).

Phosphorus availability was exceptionally high at 107.4 ppm, likely due to prior synthetic fertilizer applications. While this is beneficial, acidic soil conditions may still

Table 1
Initial soil chemical properties of paddy fields in Muara Kelantan

No	Analysis	Value	Category
1	pH H_2O	4.70	Acidic
2	Organic carbon (%)	14.3	Very high
3	Total nitrogen (%)	0.43	Moderate
4	C/N ratio	33.3	Very high
5	Available phosphorus (ppm)	107.4	Very high
6	Exchangeable bases		
	a. Ca (cmol(+)/kg)	8.18	Moderate
	b. Mg (cmol(+)/kg)	4.37	High
	c. K (cmol(+)/kg)	0.89	High
	d. Na (cmol(+)/kg)	0.13	Low
7	Cation exchange capacity (CEC) (cmol(+)/kg)	32.2	High
8	Exchangeable aluminium (Al) (cmol(+)/kg)	7.17	Moderate
9	Aluminum saturation (%)	22.27	Low
10	Base saturation (%)	42.1	High
11	Exchangeable iron (Fe) (ppm)	4881	Very high

lead to phosphorus fixation, reducing its bioavailability over time (McDowell et al., 2024). Among the exchangeable base cations, calcium (Ca) was moderate at 8.18 cmol(+)/kg, magnesium (Mg) and potassium (K) were high at 4.37 and 0.89 cmol(+)/kg, respectively, and sodium (Na) was low at 0.13 cmol(+)/kg. These values reflect adequate nutrient reserves for crop growth, although the low sodium levels indicate minimal salinity, which is favourable for rice cultivation (Fujii et al., 2018). The cation exchange capacity (CEC) was high at 32.2 cmol(+)/kg, showing the soil's potential to retain and supply nutrients effectively, despite its acidic nature.

Despite these favourable characteristics, the exchangeable aluminium concentration was 7.17 cmol(+)/kg, which poses a moderate risk of aluminium toxicity, especially at low pH. However, the aluminium saturation was relatively low at 22.27%, reducing its immediate impact on plant health (Saidi et al., 2021). Base saturation was high at 42.1%, suggesting that a significant proportion of exchange sites were occupied by beneficial cations like Ca, Mg, and K, providing partial buffering against acidity.

The most critical constraint identified was the extremely high concentration of exchangeable iron at 4881 ppm, which presents a severe risk of iron toxicity under waterlogged conditions. Such high Fe levels are typical in acidic soils subjected to flooding, where reducing conditions increase Fe solubility (Aung & Masuda, 2020; Wairich et al., 2024). Iron toxicity can severely impact rice growth by interfering with root development, nutrient uptake, and overall plant performance. These findings align with earlier studies, highlighting that while Ultisol soils may have high nutrient reserves due to prior management efforts, they remain constrained by acidity and toxic iron levels, requiring targeted management strategies to improve productivity (Noor et al., 2012).

Effects of Periodic Flooding on Soil pH

Soil pH plays a vital role in determining nutrient availability, microbial activity, and the solubility of toxic elements such as aluminium (Al) and iron (Fe). In this study, soil pH was measured at the end of the flooding phases for each treatment to assess the cumulative impact of periodic flooding on the chemical properties of Ultisol soils. The results, presented in Table 2, illustrate how different flooding and drying regimes influenced soil pH dynamics.

For continuous flooding (A0), pH remained relatively stable throughout the experiment, with values ranging from 6.27 to 7.44. This stability reflects the dominance of reducing conditions under constant submergence, which tends to increase pH by promoting the reduction of Fe^{3+} to Fe^{2+} and the accumulation of bicarbonates and other reduced compounds (Amini et al., 2022; Ponnamperuma, 1972). These conditions are characteristic of continuously flooded soils but can also lead to excessive solubility of Fe^{2+}, posing risks of iron toxicity.

In treatments involving periodic flooding and drying (A1 to A4), soil pH varied depending on the timing and duration of the flooding phases. In A1 (2 weeks flooding, 2 weeks drying), pH dropped during shorter flooding periods, reaching a low of 5.68 in week 6. The relatively acidic conditions can be attributed to the incomplete reduction of acidic compounds during the brief flooding intervals and the carryover effects of acidification from the preceding drying phases (Wang et al., 2022). In A2 (2 weeks drying, 2 weeks

Table 2
*Average soil pH under periodic flooding in Ultisol fields cultivated with rice (*Oryza sativa *L.) (n = 5)*

Week	A0	A1	A2	A3	A4
2	6.67	6.33	-	-	-
3	6.73	-	-	6.74	-
4	6.54	-	6.49	-	-
5	6.35	-	-	-	-
6	6.27	5.68	-	-	5.53
7	6.61	-	-	-	-
8	6.62	-	6.14	-	-
9	6.62	-	-	6.37	-
10	6.60	6.43	-	-	-
11	6.66	-	-	-	-
12	6.70	-	7.18	-	6.26
13	7.40	-	-	-	-

Note. Data = means

flooding), the pH increased significantly during the flooding phases, reaching 7.18 in week 12. The drying phases preceding each flooding period likely enhanced the oxidation of Fe^{2+} to Fe^{3+}, reducing the availability of soluble Fe^{2+} and consequently lowering soil acidity (Leinonen, 2023). This treatment demonstrated the most effective stabilization of soil pH among the shorter flooding-drying cycles.

The treatment with 3 weeks flooding followed by 3 weeks drying (A3) exhibited consistently stable pH levels, ranging from 6.37 to 6.74. The longer flooding intervals allowed for more complete reduction of acidic components, while the subsequent drying phases minimized the accumulation of toxic ions by promoting oxidation and precipitation of $Fe(OH)_3$ and $Al(OH)_3$. Similarly, A4 (3 weeks drying followed by 3 weeks flooding) showed pH dynamics reflecting the extended oxidation effects of longer drying phases, with the lowest observed pH of 5.53 in week 6. However, pH gradually increased during the flooding phases, indicating the ameliorative effects of periodic submergence.

Periodic flooding modulated soil pH by alternating between reducing and oxidizing conditions. During flooding phases, Fe^{3+} was reduced to Fe^{2+}, leading to a temporary decrease in acidity and an accumulation of basic compounds, while drying phases facilitated Fe^{2+} oxidation and the precipitation of insoluble hydroxides (Kögel-Knabner et al., 2010; Ninin et al., 2024). This dynamic cycle contributed to pH stabilization in Ultisols, with treatments like A2 and A3 showing the greatest potential for improving soil conditions for rice cultivation (Saidi et al., 2021). However, as pH was measured only at the end of the flooding phase, this study primarily captured the cumulative effects of prolonged reduction but did not fully account for fluctuations during the drying phase. Since redox conditions continue to evolve throughout both phases, future research should incorporate real-time pH monitoring across the entire flooding-drying cycle to better understand short-term variations in Fe solubility and their implications for long-term soil chemistry (Wu et al., 2019). Despite this limitation, these findings suggest that periodic flooding and drying can serve as a promising strategy for stabilizing soil pH and mitigating Fe toxicity in newly cultivated Ultisols, contributing to more sustainable rice production on marginal soils.

Effects of Periodic Flooding on Fe Dynamics

Periodic flooding significantly alters iron (Fe) dynamics in Ultisol soils, which are commonly characterized by high levels of exchangeable Fe and acidic conditions. In this study, Fe^{2+} concentrations and soil pH were assessed under different flooding and drying regimes to evaluate their effects on mitigating iron toxicity, a critical challenge in rice cultivation on these marginal soils. Figure 2 illustrates the effects of periodic flooding on Fe concentrations in floodwater under different treatments.

Continuous flooding (A0) maintained Fe^{2+} concentrations at an estimated average of 7.49 ppm, indicating stable but strongly reducing conditions that favoured the accumulation

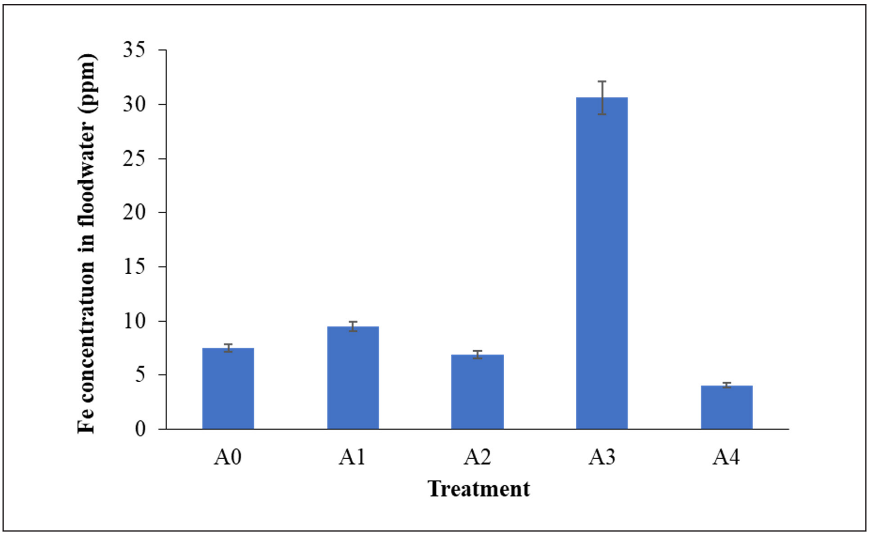

Figure 2. Effects of periodic flooding on Fe dynamics (*n* = *5*)

of soluble Fe^{2+}. This concentration was higher than in periodically drained treatments, suggesting sustained Fe^{3+} reduction without intermittent oxidation. The relatively stable pH range of 6.27 to 7.44 reflects bicarbonate formation and continuous Fe^{3+} reduction under anaerobic conditions (Amini et al., 2022). Although periodic drying phases in other treatments led to Fe^{2+} reoxidation, the absence of such fluctuations in A0 resulted in prolonged Fe^{2+} availability. This persistent presence of soluble Fe^{2+} under continuous flooding poses risks of chronic iron toxicity, as rice roots remain exposed to excessive iron concentrations for an extended duration (Aung & Masuda, 2020; Wairich et al., 2024).

Periodic flooding with shorter drying intervals, such as in treatment A1 (2 weeks flooding followed by 2 weeks drying), resulted in an Fe^{2+} concentration of 9.49 ppm by the end of the experiment. This increase can be attributed to incomplete oxidation of Fe^{2+} during the brief drying phases, which limited the conversion of soluble Fe^{2+} into insoluble Fe^{3+}. The pH in A1 also dropped as low as 5.68, further increasing Fe solubility under acidic conditions (Saleem et al., 2023). These findings suggest that shorter drying intervals may be insufficient to effectively mitigate iron toxicity in soils with high Fe levels.

In contrast, treatment A2, which involved 2 weeks of drying followed by 2 weeks of flooding, led to a more controlled Fe^{2+} concentration of 6.92 ppm. The longer drying intervals allowed for enhanced oxidation of Fe^{2+}, reducing its solubility and toxicity during subsequent flooding phases. This treatment also stabilized soil pH, which peaked at 7.18 during flooding, suggesting that alternating aeration and reduction cycles contributed to both iron detoxification and improved soil buffering capacity (Rupngam & Messiga, 2024).

Interestingly, treatment A3, which extended the flooding duration to 3 weeks followed by 3 weeks of drying, exhibited the highest Fe^{2+} concentration at 30.6 ppm, demonstrating that prolonged reducing conditions promote excessive Fe^{2+} solubilization. While pH remained relatively stable (6.37–6.74), the extended flooding phase caused Fe^{2+} accumulation to exceed levels observed in other treatments, increasing the risk of chronic iron toxicity (Saidi et al., 2021).

The most notable results were observed in treatment A4, which involved 3 weeks of drying followed by 3 weeks of flooding. This treatment recorded the lowest Fe^{2+} concentration among the periodic flooding regimes, with an average of 4.08 ppm. The extended drying phases provided sufficient time for the oxidation of Fe^{2+} to Fe^{3+}, forming insoluble precipitates such as $Fe(OH)_3$ that significantly reduced the solubility of iron during flooding. The corresponding pH dynamics in A4, with a low of 5.53 during early phases but increasing during flooding to stabilize around 6.26, reflect the ameliorative effects of periodic oxidation and the accumulation of bicarbonates during reducing conditions (Karimian, 2017).

When compared to the initial soil characteristics, the results demonstrate the transformative impact of optimized water management on iron dynamics and pH stabilization. The initial pH of 4.70 and exchangeable Fe concentration of 4881 ppm highlight the inherent challenges of cultivating rice in Ultisol soils. Treatments like A4 effectively reduced Fe^{2+} concentrations to levels well below the initial soil conditions, while also elevating the pH to near-neutral levels. This improvement underscores the importance of balancing reducing and oxidizing conditions through extended drying intervals to mitigate iron toxicity and enhance soil health.

The findings of this study align with previous research emphasizing the role of periodic flooding in managing iron solubility in paddy soils (Saidi et al., 2021). Beyond its direct effects on Fe^{2+} solubility and pH stabilization, periodic flooding offers potential long-term environmental benefits. Compared to continuous flooding, periodic drying phases reduce methane (CH_4) emissions, a major greenhouse gas associated with anaerobic decomposition in flooded paddy fields (Wassmann et al., 2020). Additionally, alternating wet and dry cycles can enhance soil aeration, promoting microbial activity that supports nutrient cycling and long-term soil fertility. These benefits suggest that optimized flooding regimes not only improve rice productivity but also contribute to more climate-resilient and sustainable agricultural practices. The results underscore the need for future research on the cumulative effects of periodic flooding across multiple cropping cycles, particularly in marginal tropical soils where sustainable water management is crucial for maintaining soil health and reducing environmental footprints.

Responses of Growth and Yield Parameters of Rice (*Oryza sativa* L.)

Periodic flooding profoundly influenced the growth and yield parameters of rice cultivated on newly opened Ultisol soils, characterized by an acidic pH (4.70), high organic carbon content (14.3%), and elevated levels of exchangeable iron (4881 ppm). These inherent soil constraints necessitate careful water management strategies to mitigate iron toxicity and enhance rice productivity. The effects of different flooding and drying intervals on plant height, tiller number, panicle number, grain quality, and dry weights are summarized in Table 3.

The periodic flooding regimes significantly affected plant growth and productivity, as reflected in the data from Table 3. The initial soil characteristics of high acidity and Fe toxicity likely limited plant performance under continuous flooding (A0), which produced relatively low plant height (117.66 cm), tiller number (6.30), and panicle number (6.20). These findings suggest that constant submergence exacerbated reducing conditions, leading to higher Fe^{2+} solubility in the water column (2.01 ppm) and reduced oxygen availability, thereby restricting root growth and nutrient uptake (Ponnamperuma, 1972).

The most favourable results were observed in treatment A1, where alternating 2-week intervals of flooding and drying produced the tallest plants (126.80 cm), the highest tiller number (7.80), and the greatest panicle number (7.40). This treatment also resulted in the highest grain yield, with dry grain weight (DGW) reaching 25.0 g and milled grain weight (MGW) at 19.20 g. The shorter drying intervals in A1 likely facilitated the oxidation of Fe^{2+} to Fe^{3+}, reducing its solubility and toxicity while maintaining adequate soil moisture for optimal plant growth (Karimian, 2017). The relatively high percentage of filled grains (77.10%) in A1 further underscores the efficacy of this water management strategy in balancing vegetative and reproductive growth phases.

Conversely, treatment A2, which involved 2 weeks of drying followed by 2 weeks of flooding, resulted in significantly lower productivity. The dry grain weight (DGW) was only 13.9 g, the lowest among all treatments, and the percentage of filled grains dropped to 59.50%. These results suggest that the alternating water regime in A2 disrupted nutrient availability during critical growth phases, emphasizing the importance of precise water

Table 3
*Growth and yield parameters of rice (*Oryza sativa *L.) under periodic flooding (n = 5)*

Treatment	Plant Height (cm)	Tiller Number	Panicle Number	% Filled Grains	Dry Grain Weight (g)	Milled Grain Weight (g)	Weight of 100 Grains (g)
A0	117.66 b	6.30 a	6.20 a	75.20 b	23.8 c	17.40 c	19.00 a
A1	126.80 c	7.80 b	7.40 b	77.10 b	25.0 c	19.20 c	19.50 a
A2	117.41 b	7.00 ab	6.00 a	59.50 a	13.9 a	11.10 a	17.60 a
A3	120.50 b	7.50 b	6.70 ab	65.20 ab	18.8 b	14.50 b	18.10 a
A4	109.80 a	7.30 ab	6.70 ab	67.37 ab	17.2 ab	14.31 b	17.50 a

Note. Numbers followed by different letters in the same column indicate significant differences (DMRT, $p < 0.05$).
Data = means

management in high-Fe soils. Treatments with extended flooding or drying phases (A3 and A4) demonstrated mixed outcomes. While A3 achieved moderate plant height (120.50 cm) and tiller production (7.50), the prolonged flooding intervals likely increased Fe^{2+} solubility (30.6 ppm), negatively affecting yield components such as dry grain weight (18.8 g DGW) and percentage of filled grains (65.20%). Similarly, A4, with its extended drying phases, recorded the lowest plant height (109.80 cm) and reduced grain quality (67.37% filled grains and 14.31 g MGW), indicating that longer drying periods induced temporary moisture stress that offset the benefits of Fe detoxification.

These findings highlight the critical role of balanced flooding and drying intervals in mitigating Fe toxicity while maintaining soil moisture and nutrient availability. The effectiveness of A1 in enhancing growth and yield aligns with previous studies that emphasize the importance of periodic flooding in managing iron solubility and toxicity in paddy soils (Saidi et al., 2021). Beyond improving soil conditions and rice productivity, periodic flooding offers additional benefits for sustainable water management, as it reduces the need for continuous irrigation and helps optimize freshwater use, an increasingly critical factor in rice-growing regions facing climate variability and water scarcity (Ninin et al., 2024). The ability of this method to sustain high yields on Fe-toxic soils also has important food security implications, particularly in Southeast Asia, where rice is a staple crop for millions of smallholder farmers (Tan et al., 2019; Wu et al., 2019). Future research should explore the long-term effects of periodic flooding on soil nutrient dynamics, microbial activity, and scalability across diverse agroecosystems, ensuring that this approach can contribute not only to iron toxicity mitigation but also to climate-resilient and water-efficient rice farming systems on marginal soils.

CONCLUSION

This study demonstrates that periodic flooding significantly influences the growth and yield of rice (*Oryza sativa* L.) on newly opened Ultisol soils, which are characterized by high acidity (pH 4.70) and elevated Fe^{2+} concentrations (4881 ppm). Continuous flooding (A0) led to reduced growth and yield due to persistent iron toxicity, while the two-week flooding–two-week drying (A1) treatment produced the highest rice productivity, with improvements in plant height, tiller number, and dry grain yield. The alternating flooding-drying cycle in A1 effectively regulated Fe^{2+} solubility, stabilized pH, and mitigated iron toxicity, creating a more favourable soil environment. However, extended flooding (A3) resulted in excessive Fe^{2+} accumulation (30.6 ppm), whereas prolonged drying in A4 induced moisture stress, highlighting the importance of balancing oxidation-reduction processes. This study was conducted under controlled greenhouse conditions and a single growing cycle, which may not fully capture long-term soil transformations and field-scale variability. Future research should explore multi-season field trials, assess the long-term impact of periodic flooding on soil fertility, and evaluate its economic feasibility to ensure scalability in real-world rice production systems. While periodic flooding presents a promising water management strategy for enhancing rice productivity on iron-toxic Ultisol soils, further optimization is required to maximize its benefits across diverse tropical agroecosystems.

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Phosphate Solubilizing Bacteria (PSB) and Commercial Rock Phosphate: An Effective Combination for Oil Palm Nursery

Ding Haoran, Tan Geok Hun* and Susilawati Kasim

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

ABSTRACT

The growth of oil palm trees depends on phosphate availability. Phosphate-solubilizing bacteria (PSB) enhance the availability of phosphorus from phosphate rock by its phosphorus solubilizing ability. Phosphate solubilizing bacteria (PSB) can improve the available phosphorus content provided by phosphate rock through its phosphorus solubilization ability. In the management of palm tree nurseries, it is vital to carefully choose the appropriate type and application rate of phosphate fertilizers based on the growth requirements and soil characteristics of palm saplings to achieve optimal growth outcomes. The purpose of this study was to determine how well oil palm nursery could benefit from the use of commercial rock phosphate in conjunction with phosphate-solubilizing bacteria (PSB). *Bacillus marisflavi* and *Bacillus aryabahattai* populations in 1 g of compost were found to be 2×10^9 and 1×10^8, respectively, using the plate count method. As seen by leaf count, oil palm size, and frond height, the development rate and quality of oil palm seedlings are greatly enhanced by the combination of Morocco rock phosphate (MRP) and PSB. This study demonstrated that a combination of PSB and commercial phosphate rock is beneficial to oil palm seedlings and sought to determine its efficacy in oil palm nurseries.

Keywords: *Bacillus aryabahattai*, *Bacillus marisflavi*, commercial rock phosphate, oil palm, phosphate solubilizing bacteria (PSB)

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E-mail addresses:
dinghaoran0@gmail.com (Ding Haoran)
geok_hun@upm.edu.my (Tan Geok Hun)
susilawati@upm.edu.my (Susilawati Kasim)
* Corresponding author

INTRODUCTION

The monoecious oil palm (*Elaesis guineensis* Jacq.) is a member of the *Aracaceae* family and belongs to the genus *Elaeis* (Corley & Tinker, 2015). Although it is widely grown in tropical nations like Malaysia, Indonesia, Nigeria, and Thailand, it is native to West and Central Africa. The oil palm, a tropical tree, is cultivated for its oil-rich

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fruit. (Rogers & Kadner, 2022). Fruit oil is a versatile substance that finds application in a range of industries, such as food, cosmetics, and biofuel production. Nearly 40% of all vegetable oil produced is palm oil, making it the most frequently used vegetable oil in the world (Myzabella et al., 2019; Singh et al., 2013). With contributions to global oil palm production and exports of 25.8% and 34.3%, respectively, Malaysia is the second-largest producer and exporter of the crop. The principal importers are from United States, India, the European Union, China and Pakistan (Parveez et al., 2022).

Phosphorus is an essential component for oil palm reproductive development, especially for the formation of flowers and fruits. Fruit set, size, and oil content can all be improved with an adequate supply of phosphorus (Etesami, 2019). Only a small amount of the phosphorus in the soil is available to plants, and the majority is unavailable. Phosphate is typically not able to be utilised directly by plants due to its non-bioavailability in soil. Only free, readily available forms of phosphorus can be used by plants from the soil. Soil microbes or plant roots facilitate the availability of soil phosphates (Barroso & Nahas, 2005; Richardson & Simpson, 2011).

One of the major non-renewable resources in the world, phosphorus is found in phosphate rock, a type of non-detrital sedimentary rock that is mostly formed in igneous, metamorphic, and sedimentary rocks. In most minerals, phosphorus takes the form of orthophosphate (Ouabid & Raji, 2023). There are several applications for phosphate rock, including the production of phosphate fertilisers, phosphoric acid, pure phosphorus (yellow and red), and other chemical raw materials. The primary ingredient in high-efficiency phosphate fertiliser and phosphate is phosphoric acid (Khan et al., 2022). Phosphate rock deposits are distributed in a somewhat concentrated manner; over 70% of the world's reserves are found in Morocco (Elamrani & Lemtaoui, 2016).

The group of rhizobacteria that promote plant growth (PGPR) includes phosphate solubilizing bacteria (PSB). According to Satyaprakash et al. (2017), PSBs have the capacity to solubilize phosphate, changing insoluble phosphatic compounds into soluble forms in soil that are then available for plant absorption. By dissolving and absorbing non-bioavailability P, phosphate solubilizing bacteria (PSB) transform it into bioavailability P to meet plant needs (Chen & Liu, 2019). However, the kind and number of organic acids produced determine the solubilization impact (Tucher et al., 2017).

The effectiveness of PSB in improving phosphorus availability depends on various factors, including the type and quantity of organic acids produced. Limited research has explored the combined application of commercial rock phosphate and PSB in enhancing phosphorus uptake, growth, and nutrient utilization in oil palm nurseries. This gap highlights the need to investigate the synergistic effects of rock phosphate and PSB on the growth and nutrient dynamics of oil palms, addressing both the immediate agricultural requirements and the broader sustainability challenges associated with phosphorus use.

This study investigated the effects of commercial rock phosphate and Phosphate Solubilizing Bacteria (PSB) on oil palm growth and nutrient utilisation in oil palm nurseries.

MATERIALS AND METHODS

Production Process of Compost

Various organic materials, such as decanter cake, cocoa shells, and coffee grounds were selected for the composting process. All Cosmos Industries Sdn. Bhd. supplied these organic components, which are commonly used in organic fertilizer production. A compost column was filled with twenty metric tonnes of the different kinds of organic materials that were chosen and mixed well. To speed up the breakdown process, a solution of microorganisms that break down was sprayed onto the thoroughly combined organic materials. The entire composting process took 14 days, with each compost column being stirred once a day by a self-propelled compost turner. Based on the wet weight, 10% of the two PSB bacterial inoculants ($1 \times 10^{8\text{-}9}$ cfu/ml) were uniformly sprayed into the compost on day 14.

Quality Checking of Compost Samples

We gathered compost samples from All Cosmos Industries Sdn. Bhd. As a result, the generated compost's quality was examined. The moisture content, particle size, pH, carbon to nitrogen ratio, total N, P, K, Mg, and B content, as well as total organic materials, were all measured for each sample. Using a Shimadzu moisture analyser M0C63u model, the moisture content was determined. The product's moisture content must be less than 8% (Richard et al., 2002). The compost's particle size was measured using a 3.2 mm perforated plate sieve. The acidity or alkalinity of a substance is indicated by its pH value.

In a sterile beaker, 5 g of the sample were weighed. The sample was then stirred with a glass rod and 50 mL of distilled water added. The pH metre was calibrated using three buffer solutions with pH values of 4.0, 7.0, and 10.0 prior to pH testing. The material was placed in a suspension of distilled water (5 g/50 ml) and left for two hours to measure its pH. Using the dry oxidation or combustion method and a CHNS analyser, the total organic carbon and total nitrogen were calculated. Samples were crushed, weighed, and freeze-dried before was placed in a tin capsule containing vanadium pentoxide and burned in a reactor at 1000°C. After the sample and container melted together, the tin sped up a strong reaction (flash combustion) in an oxygen-improved environment for a limited period (Ge et al., 2022).

Total P, K, Mg, and B contents were determined using the inductively coupled plasma atomic emission spectrometry method in accordance with (ICP-AES). A porcelain crucible containing one gramme of the material was filled, heated to 500°C for two hours, and then removed. After adding 3 mL of HNO_3, the sample was roasted on a hot plate at 100°C until it was dry. After being reinserted into the muffle furnace, the crucible was muffled for a further hour at 500°C. After allowing the crucible to cool, 10 mL of HCl were added.

The material was transferred and then diluted in a 50 mL volumetric flask using deionized water. AOAC Method 985.01 analysis was performed on the sample ash solution that was collected (Hemidat et al., 2018).

The total organic matter was determined using the loss-on-ignition method (Chai et al., 2013). A 2 g oven-dried sample was weighed and placed it in a silica crucible and heated it to 550°C for 24 hours in a muffle furnace. The weight difference between the initial sample, the burned sample, and the leftover ash was used to quantify the amount of organic matter.

Survival of PSB Cultures in the Compost

To create compost, two PSB cultures were introduced ($1 \times 10^{8\text{-}9}$ cfu/ml for each strain). The plate count method was used to determine the total number of PSB in the sample. The term "total viable count" describes the entire number of colonies. The 95 ml of sterile phosphate buffer saline (PBS) was placed within a conical flask, into which 10 g of fertiliser samples were weighed and dissolved. After that, the conical flask was put in a shaking incubator set to 200 rpm for ten to fifteen minutes. Sterile distilled water was used for serial dilution up to a dilution factor of 10^{-9}. For the purpose of phosphate solubilization bacteria growing media, Pikovskaya agar medium was produced. The glass hockey stick was then used to distribute the suspension across the agar plate surface. After that, plates were incubated at room temperature 27°C for up to 7 days, the colony forming units (CFU) were counted (Chung et al., 2005).

Field Study

The Tenera oil palm of three months old were planted in the field of Ladang 10, Faculty of Agriculture with different treatment (Table 1).

The size and shape of the oil palm seedlings chosen as the experimental samples were comparable. A thin layer of soil was placed inside 20 × 20 black polybags housing oil palm seedlings. Then, each group - aside from the control group—received 100 g of the matching therapy (Figure 1). Finally, soils were placed into the polybags (Chao, 2018). Vegetative measures, chlorophyll readings, period yield records, and the phosphorus content of leaves and rachis were all observed and documented as parameters for comparison across all treatments. A total of 120 oil palm seedlings were arranged in a Randomized Complete Block Design (RCBD), with five replications each treatment and six oil palm samples each replication, as shown in Figure 2.

Table 1
Treatment for oil palm nursery

Treatment	Detail of fertilizer application
T1	Control, no fertilizer
T2	Moroccan Rock Phosphate (MRP)
T3	30% Compost + Phosphate Solubilizing Bacteria (PSB) + 70% Moroccan Rock Phosphate (MRP)
T4	Compost + Phosphate Solubilizing Bacteria (PSB)

Figure 1. Oil palm seedlings in black polybags

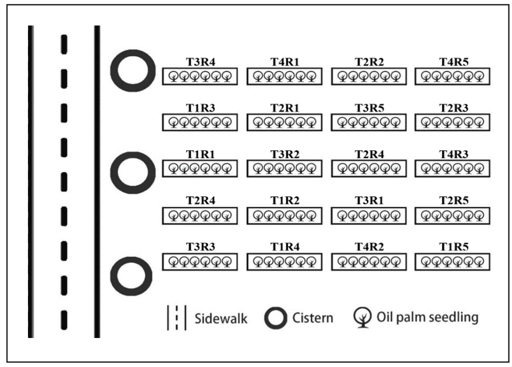

Figure 2. Experimental design

Data Analysis

Five replicates of each treatment were used in the analysis, which followed the RCBD experimental design. The Prism 9 statistical analysis system and the analysis of repeated measures ANOVA were used to analyse the field data. There were notable variations ($P<0.05$) between the treatments.

RESULTS

Formulation of Compost

The composition of the compost used in this investigation is presented in Table 2. Analysis of the compost sample revealed that the concentrations of all six heavy metals were below the detection limits of the instrument. Additionally, no *Salmonella*, *Escherichia coli*, or other pathogens were detected in the compost.

Table 2
Physicochemical and microbiological analysis of compost

Test Parameter	Unit	Result
pH	-	6.7
Electrical Conductivity	mS/cm	3.16
C/N ratio	-	15.01
Organic Matter	%	66.7
Moisture Content	%	40.1
Total Nitrogen, N	%	2.4
Phosphorus, P	%	0.2
Potassium, K	%	1.1
Calcium, Ca	%	0.6
Magnesium, Mg	%	0.3
Nickel, Ni	ppm	N.D (<0.01)
Chromium, Cr	ppm	N.D (<0.01)
Lead, Pb	ppm	N.D (<0.01)
Cadmium, Cd	ppm	N.D (<0.01)
Arsenic, As	ppm	N.D (<0.01)
Mercury, Hg	ppm	N.D (<0.01)
Escherichia coli	MPN/g	N.D (<0.01)
Salmonella	-	absent

Note. N.D mean not detected

Survival Test of Two PSB in Compost

In this study, the PSB inoculant was evenly sprayed on the compost. The results for PSB populations in the compost, which were reconfirmed, are shown in Table 3.

Table 3
PSB population in 1 g of compost

Phosphate Solubilizing Bacteria	CFU/g
Bacillus marisflavi	2×10^9
Bacillus aryabhattai	1×10^8

The Effectiveness of Phosphate Solubilizing Bacteria on Oil Palm at the Nursery

Three months after planting, the data was first gathered (3 MAP). Thereafter, planting took place for six months (6 MAP) and eight months (8 MAP). An appendix contains a summary of all the data. Three-month-old oil palm plants showed no discernible variations in the majority of the characteristics. This is explained by the fact that oil palm seedlings with comparable sizes and shapes were chosen for the experiment.

Figure 3 describes the size of the oil palm at three, six, and eight months old in relation to its vegetative growth. When the oil palm was three months old, there were no appreciable differences between the four treatments. Nevertheless, variations in growth are only discernible between the ages of 6 and 8 months.

At 6 months, oil palm size in T2, T3, and T4 was significantly greater than in the control group (T1). Notably, T3 outperformed both T2 and T4, suggesting that the treatment with 30% compost + Phosphate Solubilizing Bacteria (PSB) + 70% Moroccan Rock Phosphate (MRP) is more effective in promoting growth. A similar pattern was observed at 8 months, although T2 did not show a significant difference compared to T1.

Significant differences were observed when comparing the heights of the first and third fronds in most plot samples, with the exception of the first and third frond heights at three months, as shown in Figures 4 and 5. Regarding the height of the first frond at 6 months, T2, T3, and T4 demonstrated significantly better performance compared to the control group (T1). Interestingly, T3 outperformed T2 and T4, indicating that the treatment with 30% compost + Phosphate Solubilizing Bacteria (PSB) + 70% Moroccan Rock Phosphate (MRP) is more effective in these treatments. Similarly, at 8 months, the same trend was observed, though T2 did not show a significant difference compared to T1. Furthermore, the height of the third frond followed a similar trend in group mean scores to that of the first frond at both six and eight months.

To conclude, the oil palms in the treatment without PSB-mixed compost, aged 6 and 8 months, have shorter fronds compared to those in the treatment with it. Additionally, the treatment with MRP shows a noteworthy distinction from the group without MRP in oil palms of the same ages.

Figure 6 shows the results of chlorophyll content in oil palm leaves. At six months, a comparison of the group mean scores revealed that T1, T3, and T4 demonstrated significantly higher efficacy than T2. The chlorophyll content in the 8-month-old oil palm leaves followed the same trend. In summary, the group with only MRP showed lower values compared to the others.

Figures 7 and 8 displayed the width and thickness of the third frond data analysis for oil palm. The thickness of the oil palm third frond in both six months and eight months were found to the comparison of mean scores among groups T1, T3, and T4 showed significantly higher efficacy than T2. The six months shows the extreme significant difference which

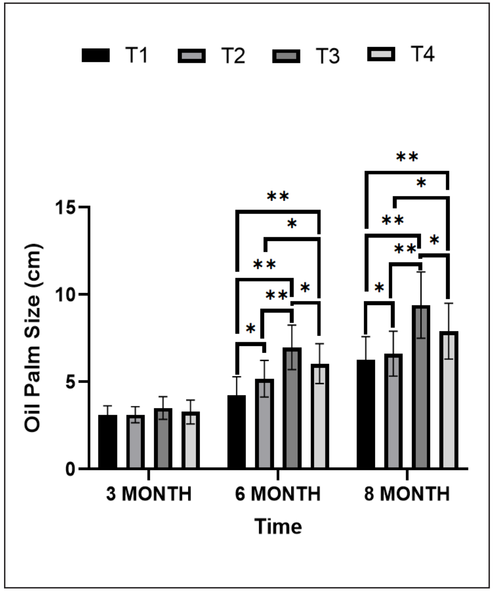

Figure 3. The size of 3 months, 6 months, 8 months old oil palm seedling
Note. Measurement was recorded the 3 months old oil palm, 6 months old oil palm and 8 months old oil palm. The repeated measured ANOVA significant difference all-pairwise comparison test. It was used to detect statistical differences among the means at P=0.05 and P=0.01 significance level. The bars were connected by zigzag line with * symbol means they show a significant difference (*: p<0.05; **: p<0.01). The lack of zigzag line represents no significant difference between treatments

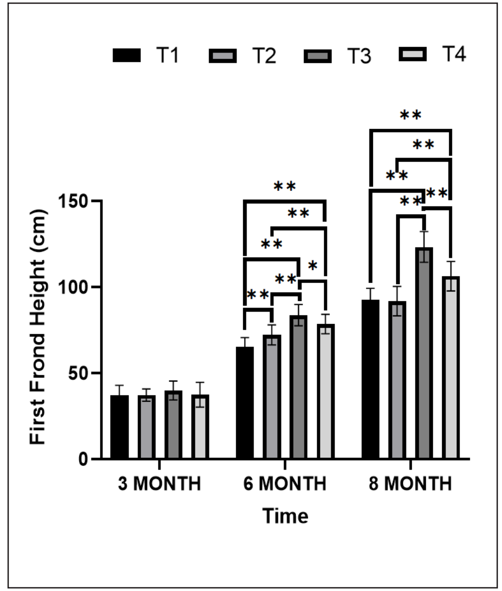

Figure 4. The height of 3 months, 6 months, 8 months old oil palm first frond
Note. Measurement was recorded the 3 months old oil palm, 6 months old oil palm and 8 months old oil palm. The repeated measured ANOVA significant difference all-pairwise comparison test. It was used to detect statistical differences among the means at P=0.05 and P=0.01 significance level. The bars were connected by zigzag line with * symbol means they show a significant difference (*: p<0.05; **: p<0.01). The lack of zigzag line represents no significant difference between treatments

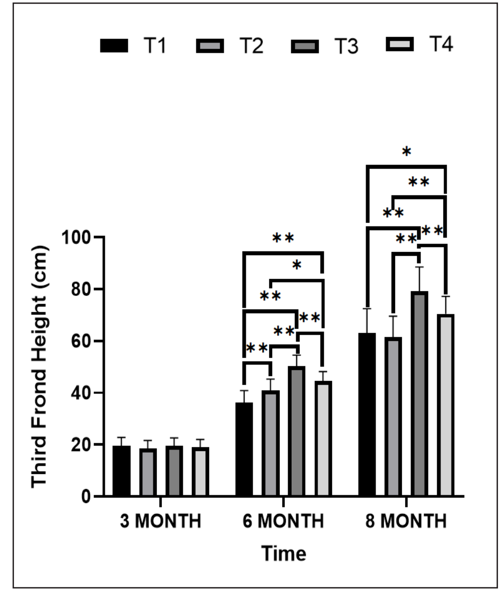

Figure 5. The height of 3 months, 6 months, 8 months old oil palm third frond
Note. Measurement was recorded the 3 months old oil palm, 6 months old oil palm and 8 months old oil palm. The repeated measured ANOVA significant difference all-pairwise comparison test. It was used to detect statistical differences among the means at P=0.05 and P=0.01 significance level. The bars were connected by zigzag line with * symbol means they show a significant difference (*: p<0.05; **: p<0.01). The lack of zigzag line represents no significant difference between treatments

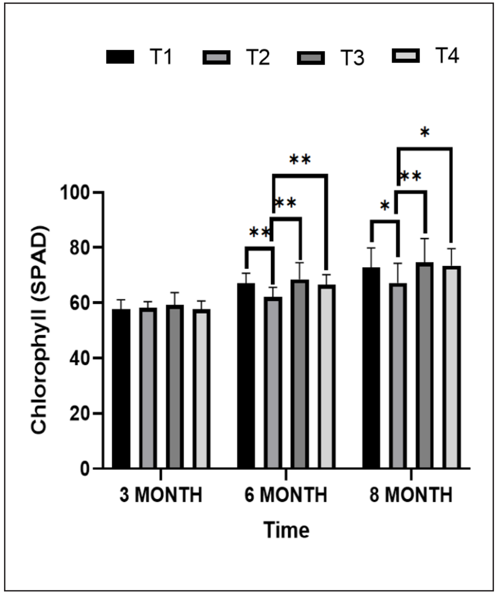

Figure 6. The chlorophyll content in 3 months, 6 months, 8 months old oil palm leaves

Note. Measurement was recorded the 3 months old oil palm, 6 months old oil palm and 8 months old oil palm by SPAD502. The repeated measured ANOVA significant difference all-pairwise comparison test. It was used to detect statistical differences among the means at P=0.05 and P=0.01 significance level. The bars were connected by zigzag line with * symbol means they show a significant difference (*: p<0.05; **: p<0.01). The lack of zigzag line represents no significant difference between treatments

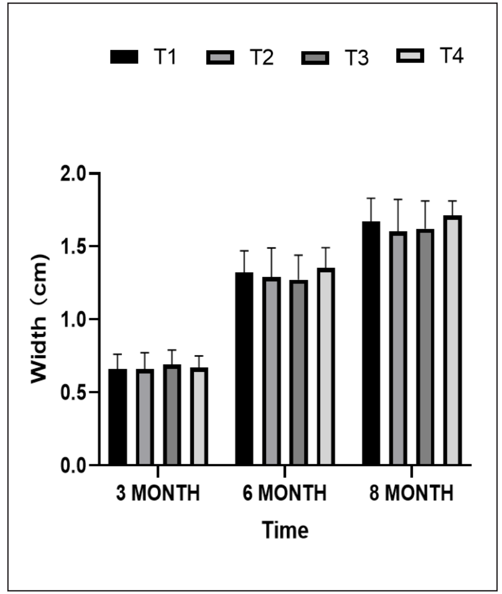

Figure 7. The width of 3 months, 6 months, 8 months old oil palm third frond

Note. Measurement was recorded the 3 months old oil palm, 6 months old oil palm and 8 months old oil palm. The repeated measured ANOVA significant difference all-pairwise comparison test. It was used to detect statistical differences among the means at P=0.05 and P=0.01 significance level. The bars were connected by zigzag line with * symbol means they show a significant difference (*: p<0.05; **: p<0.01). The lack of zigzag line represents no significant difference between treatments

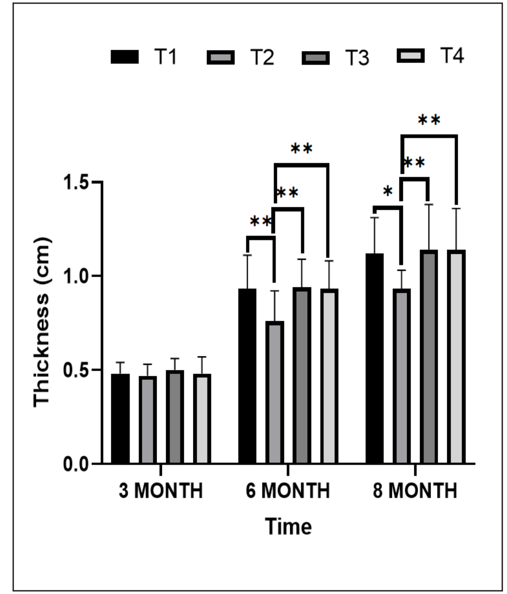

Figure 8. The thickness of 3 months, 6 months, 8 months old oil palm third frond

Note. Measurement was recorded the 3 months old oil palm, 6 months old oil palm and 8 months old oil palm. The repeated measured ANOVA significant difference all-pairwise comparison test. It was used to detect statistical differences among the means at P=0.05 and P=0.01 significance level. The bars were connected by zigzag line with * symbol means they show a significant difference (*: p<0.05; **: p<0.01). The lack of zigzag line represents no significant difference between treatments the p-value less than 0.01, however the thickness of the eighth-month oil palm third frond only exhibited a p-value less than 0.05. The width of the oil palm's third frond, however, did not provide a noteworthy outcome.

The significant increase in frond thickness in treatments with RP and PSB (T1, T3, T4) is indicative of better plant health, enhanced nutrient availability, and overall improved growth, especially at the early stages of development (six months). This supports the hypothesis that frond thickness is a key indicator of the plant response to fertilizer treatments, which can influence its long-term productivity and oil yield. The lack of significant results for frond width further suggests that thickness is a more reliable measure of plant efficacy in this context.

Only after six months did the quantity of oil palm leaf significantly different in Figure 9. The group mean score comparison results with evident differences were the effectiveness of T2, T3, and T4 was significantly greater than that of control group (T1). T3 which possesses PSB and MRP showed significantly higher efficacy than T2 and T4 which only possess single ingredient. There are observed significant differences among all the treatments in 6 months old oil palm trees.

Eight-month-old oil palm trees were divided into rachises and leaves after eight months of growth. After drying, these plant portions were transported to the laboratory for a study of their phosphorus content. The findings of the laboratory analysis are displayed in Figure 10. There were no notable variations found between the other treatment groups, with the exception of the phosphorus content discrepancy between the rachises of T1 and T3. Variations in phosphorus absorption levels could be the cause of the notable differential in phosphorus concentration between T3 and T1 in the rachises.

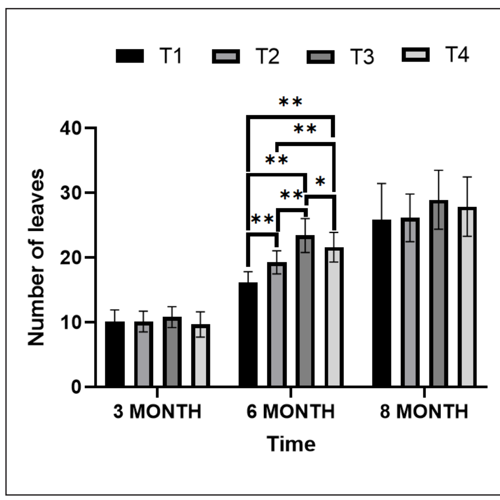

Figure 9. The number of 3 months, 6 months, 8 months old oil palm leaves

Note. Measurement was recorded the 3 months old oil palm, 6 months old oil palm and 8 months old oil palm. The repeated measured ANOVA significant difference all-pairwise comparison test. It was used to detect statistical differences among the means at P=0.05 and P=0.01 significance level. The bars were connected by zigzag line with * symbol means they show a significant difference (*: $p<0.05$; **: $p<0.01$). The lack of zigzag line represents no significant difference between treatments

DISCUSSION

Survival Test of Two PSB in Compost

Table 3 shows that there were two PSBs in 1 g of compost, with respective populations of 2×10^9 and 1×10^8. It demonstrates that two

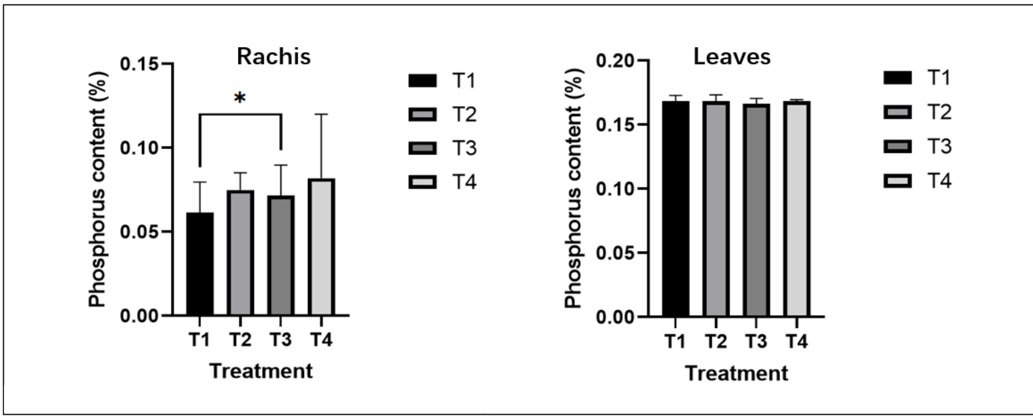

Figure 10. The phosphorus content of 8 months old oil palm rachis and leaves *Note*. Analysis data was from the dried rachis and leaves of 8 months old oil palm. The repeated measured one-way ANOVA significant difference all-pairwise comparison test. It was used to detect statistical differences among the means at P=0.05 significance level. The bars were connected by zigzag line with * symbol means they show a significant difference (p<0.05). The lack of zigzag line represents no significant difference between treatments

PSBs could live and procreate in compost. This suggests that composting offers the PSBs the perfect habitat for growth and proliferation. Additionally, this research might provide insightful information for managing and optimising composting operations. Bacteria that solubilize phosphates can break down and make use of phosphorus molecules. They have the ability to change organic phosphorus into inorganic forms, increasing compost's phosphorus concentration. This gives compost a higher nutritional content and gives plants the phosphorous minerals they need. Understanding these microbes' functions in their natural habitats and how they interact with other creatures depends on this research (Timofeeva et al., 2022). PSB are essential environmental microbes that have strong ecological flexibility in their natural environments and a wide range of potential uses in the fields of biological control and environmental restoration. They also aid in the breakdown of organic contaminants, the synthesis of antibiotics, and the decomposition of organic materials (Numan et al., 2018). Compost maturation can be accelerated by modifying the growth and reproduction of phosphate-solubilizing bacteria through manipulation of composting parameters, including temperature, moisture, and ventilation.

Field Data Analysis

The oil palm seedlings treated with MRP and PSB compost in our experiment showed noticeably quicker growth rates and outperformed the control group. Malhotra et al. (2018) claimed the ability of phosphorus to promote early branching and budding, accelerate the growth of plant stems and roots, and enhance seed germination. Oil palms absorb phosphorus most quickly in their seedling stage, therefore a phosphorus deficit at this time can negatively impact the oil palm's ability to expand in the future (Ajeng et al., 2020; Lovelock et al., 2006). According to Abidemi et al. (2006), most seedlings' sizes were considerably enlarged when they were given phosphorus biofertilizer. An essential nutrient for plant growth is phosphorus. Khattab et al. (2019) showed Plants that are poor in phosphorus display signs like stunted growth, short stature, delayed production of flower buds, and increased fruit and blossom drop. Variations in the absorption of total phosphorus could affect the height of the fronds. The results of Kumar et al. (2020) also demonstrated that seed germination, as well as other plant parameters such as height and weight, significantly increased in plants treated with PSB. Additionally, the frond height of the control group, which includes MRP and PSB at 6 and 8 months, shows a significant difference compared to the other groups. This indicates that the combination of MRP and PSB positively impacts frond height in oil palms. The results also show that treatments with MRP and PSB significantly improved chlorophyll content, frond thickness and highest leaf quantity in oil palm at early stages of development, outperforming other treatments. These findings align with research by Etesami (2019), which highlighted the positive impact of PSB on nutrient availability and plant growth.

Plants with phosphorus deficit may develop without branches and shed their leaves early. Heppell et al. (2015) proved older leaves close to the base of the stem exhibit the first signs of phosphorus deficit, which then spread upward. This could help to explain the notable variations in all treatments that were seen in oil palm plants that were six months old. Plants typically contain between 0.1% and 0.5% of phosphorus in their dry weight. The movement of growth centres inside a plant and metabolic activities are intimately linked to the dispersion of phosphorus. Phosphorus is predominantly found in young shoots and root tips during the vegetative growth phase, which contributes to the plant's pronounced apical dominance (Gaiero et al., 2020; Viegas et al., 2021). This could account for why leaves have more phosphorus than rachises. Rachises sustain leaves, flowers, and fruits inside a defined area in addition to acting as a conduit for nutrients and water. Stems can occasionally carry out photosynthesis, store nutrients, and aid in reproduction. As a result, the rachises can show differences in their amounts of phosphorus absorption.

CONCLUSION

By phosphate solubilization, phosphate-solubilizing bacteria increase the amount of phosphorus that is available in the soil. They greatly raise the soil's total phosphorus levels when paired with MRP. Phosphate solubilizing bacteria (PSB) and Morocco rock phosphate (MRP) work in concert to promote oil palm seedling growth, which improves growth rates and quality. In addition, it improves soil conditions and increases oil palms' ability to absorb and utilise phosphorus. As a result, using MRP and PSB together while planting oil palms works well to improve the yield and quality of the oil palm. *Bacillus* *marsflavi* and *Bacillus aryabhattai* are the two PSB cultures which showed a high rate of survival. The benefits in terms of leaf count, oil palm size, and frond height are enhanced effectively when Morocco rock phosphate (MRP) and phosphate solubilizing bacteria (PSB) are combined. Oil palm seedlings have been shown to benefit from commercial production.

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Impact of Elevated Carbon Dioxide and Planting Density on the Growth and Physiological Responses of *Stevia rebaudiana* Bertoni

A Abzar[1], Siti Zaharah Sakimin[1,2]*, Hawa ZE Jaafar[2], Nor Elliza Tajidin[1,2], Zulkarami Berahim[2] and Mohd Firdaus Ismail[1]

[1]*Department of Crop Science, Faculty of Agriculture, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[2]*Institute of Tropical Agriculture, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

ABSTRACT

Stevia rebaudiana Bertoni produces steviol glycosides (SGs) which are 300-400 times sweeter than table sugar, non-caloric in nature, and used by diabetic patients worldwide. Increasing level of carbon dioxide (CO_2) in the atmosphere, due to environmental pollution and climate change, have the potential to influence crop growth and productivity including stevia. Additionally, planting density is another important agronomic factor that affects crop yield, but its interaction with CO_2 levels in stevia has not been adequately studied. Therefore, the current study was conducted with the objective to investigate the effects of elevated CO_2 (eCO_2) under different planting densities on the growth and physiological responses of *Stevia rebaudiana*. A factorial (2×3) experimental design was employed, with two CO_2 levels (400 ppm as ambient CO_2 [aCO_2] and 1200 ppm as eCO_2) and three planting densities: high-density vertical (HDV), high-density horizontal (HDH), and low-density horizontal (LDH). Growth data were collected monthly until the final harvest, while physiological parameters were recorded at 1st and 3rd month after planting (MAP). The results indicated that eCO_2 significantly enhanced plant growth, with the highest plant height (77.1 cm) observed in eCO_2-treated plants compared to aCO_2 (66.5 cm) at final harvest. Under aCO_2, HDH and HDV treatments resulted in taller plants than LDH at 1st MAP, but no significant differences were found at later stages. eCO_2-treated plants also showed increased branching (25%–28% more at 1st–4th MAP) compared to aCO_2-treated plants. Photosynthesis rates were 36% and 42% higher in eCO_2 plants at the 1st and 3rd MAP, respectively. LDH plants demonstrated better overall physiological performance, including higher photosynthetic rates and water use efficiency. In conclusion, eCO_2 significantly improves stevia growth and

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E-mail addresses:
abzar.khan1@gmail.com (A Abzar)
szaharah@upm.edu.my (Siti Zaharah Sakimin)
hawazej@upm.edu.my (Hawa ZE Jaafar)
elliza.tajidin@upm.edu.my (Nor Elliza Tajidin)
zulkerami@upm.edu.my (Zulkarami Berahim)
mohd.firdaus@upm.edu.my (Mohd Firdaus Ismail)
* Corresponding author

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physiology, with LDH and HDH densities showing superior performance. These results suggest that optimizing both CO_2 levels and planting density can improve stevia productivity, particularly under future climate conditions.

Keywords: Antioxidant, natural sweetener, rebaudiosides, steviosides, stomatal conductance

INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni) is a perennial herbaceous plant species which belong to the family of Asteraceae, native to specific regions of South America, specifically Brazil and Paraguay (Geuns, 2007). However, the cultivation of Stevia has expanded globally, with significant production now occurring in countries like Argentina, Japan, China, Korea, Russia, Mexico, Indonesia, the United States, Malaysia, Tanzania, Canada and Thailand (Alvarez-Robles et al., 2016; Lemus-Mondaca et al., 2012; Sumon et al., 2008). Stevia produces special secondary metabolites which are known as steviol glycosides (SGs), that are unique to *Stevia rebaudiana*, distinguishing it from other plant species (Brandle et al., 1999). It has been reported that some of the steviol glycosides derived from Stevia are 300–400 times sweeter than sucrose (Moraes et al., 2013; Yadav et al., 2011), and these compounds are non-caloric, making them suitable for consumption by individuals with diabetes, as they do not affect blood glucose levels (EFSA, 2010; Moraes et al., 2013).

The Earth's climate has changed drastically because of human activities since the pre-industrial revolution. The earth's temperature is increasing, causing global warming due to increasing concentrations of greenhouse gases, such as CO_2, CH_4, and N_2O, in the atmosphere that traps the sun rays (Abzar et al., 2017; Solomon et al., 2009). The massive generation of greenhouse gases from rapidly expanding industrial and residential sectors has caused environmental contamination (Ramanathan & Feng, 2009; Tian, 2015). Environmental pollution has caused the concentration of CO_2 in the atmosphere to rise to 408 (ppm), from 280 (ppm) at the beginning of the twentieth century before the industrial revolution (Dong et al., 2018). The Intergovernmental Panel on Climate Change (IPCC) indicated that, before the end of this century, the CO_2 concentration will cross 1,000 (ppm) (Dong et al., 2018). Elevated CO_2 is a key component of climate change, with potential implications for plant growth and productivity. Generally, increased CO_2 concentrations enhance photosynthetic rates by providing more substrates for photosynthesis. This can lead to improved biomass accumulation and altered plant physiology (Thompson et al., 2017). Studies have shown that elevated CO_2 can increase the net photosynthetic rate in various crops (Ainsworth & Long, 2005; Leakey et al., 2009). For stevia, higher CO_2 levels might boost growth and sweetener yield, as enhanced photosynthesis can result in greater production of bioactive compounds (Ziska et al., 2016). Additionally, elevated

CO_2 can affect nutritional quality and secondary metabolite production in plants. For instance, changes in CO_2 levels may affect the concentration of steviol glycosides, which are responsible for the sweetness of stevia (Lemus-Mondaca et al., 2012). Understanding these effects is essential for optimizing the economic and nutritional values of stevia under changing atmospheric conditions.

Besides environmental factors, agronomics practices such as planting density also influencing plant growth and productivity. Zhang et al. (2021) reported that higher planting densities can lead to increased competition for resources, such as light, water, and nutrients, which might affect plant morphology, yield, and overall health. However, optimal planting densities can maximize space utilization and improve yield per unit area. Research on planting densities indicates that there is an optimal density range for various crops in which growth and productivity are maximized. For Stevia, adjusting the planting density can affect leaf production, stem diameter, and the concentration of sweet compounds. Dense planting may lead to increased shading and reduced leaf area, potentially affecting photosynthetic efficiency and steviol glycoside content (Kumar et al., 2019; Gomes et al., 2018). Conversely, well-spaced plants may have better access to resources, potentially enhancing their growth and sweetener yield. The recommended planting densities for obtaining higher yield and biomass is between 83,000 and 111,000 plants/ha (Kumar et al., 2014; Madan et al., 2010; Serfaty et al., 2013). Some authors have reported that higher stands may still provide biomass increases by area (Kumar et al., 2014). The ideal plant density for stevia varies greatly, depending on the climate and soil fertility of the growing region. The current study was conducted with the aim to investigate the impact of elevated CO_2 under different planting densities on physiology and growth of *Stevia rebaudiana* under Malaysian environmental condition. In more specific terms the study aims to understand that elevated CO_2 concentration due to the changing climatic condition affects the biomass accumulation, photosynthetic rate and production of steviol glycosides which are responsible for sweetness in stevia. Furthermore, the research explores how different planting densities affect stevia leaf production, growth and yield of the sweetener.

The significance of the study lies in its potential to improve the cultivation of stevia in response to changing environmental conditions, especially in Malaysia where the cultivation of stevia is not very common. By exploring the mutual effects of elevated CO_2 and planting densities, the study focuses on understanding how to improve the production of stevia, ensuring that its growth and the productivity of valuable bioactive compounds can be maximized under local environmental and agricultural conditions. This research could further contribute to enhancing stevia's economic and nutritional value, specifically as a non-caloric sweetener suitable for individuals with diabetes.

MATERIAL AND METHODS

Experimental Site and Planting Materials

The experiment was conducted under glass house conditions located in Putra agricultural centre (PAC) University Putra Malaysia (UPM) and Tenaga National Berhad Research Centre (TNBR) Kawasan Institusi Penyelidikan, Jalan Ayer Itam, Kajang, Selangor. The glasshouse with elevated CO_2 was designed in such a way that plants could receive a 12/h photoperiod throughout the growing season. The fully sealed 5 m × 3.67 m glasshouse was continually supplied with 99.8% pure CO_2 from high-pressure CO_2 cylinders for two hours a day, from 8:00 to 10:00 a.m. via a pressure regulator. During the CO_2 exposure time, the CO_2 concentrations were measured using air-sensing CO_2 sensors assigned to each chamber. The level of CO_2 inside the glasshouse increased from 400 (ppm) to 1200 (ppm) at two-week intervals, with an elevation of 400 (ppm) each time. The greenhouse was equipped with dripped fertigation for irrigation purposes. Seedlings were prepared by stem cutting. The seedlings with height of 7–8 cm were transferred to a medium containing coco-peat without soil in 16 cm × 16 cm (16 × 16) polyethylene bags. The temperature was maintained between 27 and 35°C during the day and between 18 and 21°C at night. The relative humidity was maintained at 50-60%. To allow the plant to grow under their natural environment stevia plants were grown under 50-60% shade (light intensity 225±50 μmol m^{-2} s^{-1}) using black netting.

Experimental Design

A nested design with RCBD in four blocks was performed to conduct the current experiment. The arrangement was made on factorial bases with two factors and three levels for each of the factor. Factor a representing two CO_2 levels (400 ppm) and (1200 ppm) while factor b represented three planting densities, high-density vertical (HDV) 78 plants/meter square, high-density horizontal (HDH) 25 plants/meter square and low-density horizontal (LDH) 12 plants/meter. Factor a (CO_2) was applied across the block, while densities were nested within factor a (CO_2).

Growth Attribute

Growth-related data such as plant height, number of branches, and number of leaves were assessed monthly starting from the first month of growth until the final harvest at month four. The plants standing upright were selected from the top of the coco-peat level of the polybag to the highest point of the plant to measure plant height and were expressed in centimetres (cm) using measuring tape. Primary, secondary, and tertiary shoots were considered the number of branches. The number of branches and leaves was counted visually.

Leaf Gas Exchange

Physiological characteristics, such as photosynthetic rate (µmol CO_2 m^{-2} s^{-1}), stomatal conductance (µmol CO_2 m^{-2} s^{-1}), transpiration rate (µmol H_2O m^{-2} s^{-1}), intercellular CO_2 (µmol CO_2 m^{-2} s^{-1}), and water use efficiency ((µmol CO_2 mol-^{1} H_2O), were examined by selecting healthy leaves at the completion of the first and third growing months. All of the above-mentioned parameters were measured using a portable infrared gas analyser (Li-Cor 6400, LI-Cor Inc., Lincoln, USA) adjusted at 400 µmol mol^{-1} CO_2, 1000 µmol m^{-2} s^{-1} irradiance and saturated light condition (solar radiation > 1200 µmol. m^{-2} s^{-1}) on the abaxial surface of a leaf that has fully grown from the plant's tip. The ratio of photosynthetic rate to transpiration rate was used to calculate water use efficiency, following the methods of Condon et al. (2004).

Chlorophyll Estimation

Destructive method suggested by Lichtenthaler and Bushman (2001) were used for the estimation of Chlorophyll a, b, and total chlorophyll. Fresh and healthy leaves were collected from stevia plants (0.1 g), chopped and mixed with 10 mL of (80%) acetone in a flacon tube. To prevent light penetration into the samples the flacon tube was wrapped in aluminium foil and kept under dark conditions for 72 h. After 72 hours the solution was transferred to another tube and chopped leaves were allowed to settle. Absorbance for chlorophyll a, b and total chlorophyll was measured at 663.2, 646.8, and 470 nm using a spectrophotometric reader. For blank solution (80%) acetone was used, and the content of chlorophyll was expressed as µg mg^{-1} of fresh weight with the help following relationships:

$$\text{Chlorophyll a } (\mu g\ ml^{-1}) = (12.25 \times A663.2 - 2.79 \times A646.8)$$

$$\text{Chlorophyll b } (\mu g\ ml^{-1}) = (21.50 \times A646.8 - 5.1 \times A663.2)$$

$$\text{Total chlorophyll } (\mu g\ ml^{-1}) = (7.15 \times A663.2 + 18.71 \times A646.8)$$

Statistical Analysis

For every treatment, four replicates were created, and the results were presented as mean values. Significant variations in plant growth and physiology between the various treatments were found at a 5% probability using analysis of variance (ANOVA) and the Least Significant Difference (LSD) test.

RESULTS

Plant Height

The results from our experiment for plant height are shown in Figure 1a, which indicates that regardless of the planting density, the CO_2 treatment significantly increased the plant height consistently from the first month of planting (MAP) until the 4th MAP at the final harvest. At first MAP, the highest plant height (39.8 cm) was recorded for plants grown under elevated CO_2 (eCO_2), whereas plants grown under ambient CO_2 (aCO_2) only showed (27.6 cm) height. Following the same pattern, plant height was 16.5% higher in the 2nd month and 17% higher at 3rd MAP under eCO_2 than in plants grown under aCO_2 levels. After four MAP at the final harvest, the maximum height (77.1 cm) for stevia plants was recorded for eCO_2 treated plant compared with plants under aCO_2 (66.5 cm). Under aCO_2, different planting densities were found to be significantly different in terms of plant height at the early growth stage; however, at maturity, no significant differences were observed among the plants. In the first month, the plants under LDH showed the highest plant height (29.8 cm), followed by HDV (27.8 cm), while the HDH plants were seen with (25.2 cm) only. In contrast to month one the maximum plant height was recorded for plants grown under HDV (44.9 cm) while LDH was recorded with (44.2 cm) and HDH (44.5 cm) at 2nd month respectively. On the 3rd and 4th MAP, no significant difference in plant height was observed between all three densities. Under eCO_2, the pattern was observed to be the same as that under aCO_2 for different planting densities. However, the LDH plants showed significantly higher plant height in the 1st and 2nd months. At 1st MAP, LDH plants showed the highest plant height (42.6 cm), followed by HDV (41.2 cm) and HDH (35.5 cm). At 2nd MAP the LDH again was seen with highest plant height (56.5 cm), HDV (54.6 cm) and (46.5 cm) height was recorded for HDH plants. No significant difference was observed in the 3rd and 4th month among plants under different densities (Figure 1b).

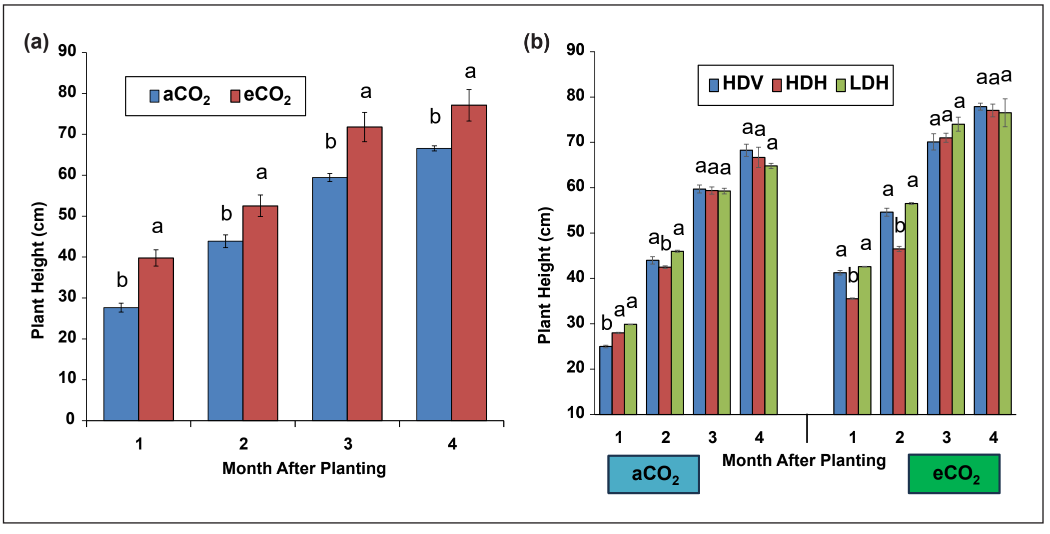

Figure 1. Plant height differences: (a) Between aCO_2 and eCO_2; (b) at different planting densities
Note. According to LSD, group means with the same letter do not differ significantly at $P \leq 0.05$. Error bars have been used to display the standard error of the mean

Number of Branches

The results from the current study show that elevated CO_2 treatment positively influenced the number of branches per plant, regardless of density. In the first month, the plants under eCO_2 produce (11.14) branches while the non-CO_2 treated plant produces (8.22) branches per plant. Similarly, the plants under eCO_2 were found to produce a 20% higher number of branches per plant at 2nd MAP, 30% at 3rd MAP, and 28% at month four than plants under aCO_2 levels (Figure 2a). Under aCO_2 levels, the HDH and HDV plants produced (9.2) and (8.2) branches per plant, respectively, which were significantly higher than LDH (7.2) in the first month. There was no significant difference recorded for plants under different densities at aCO_2 concentration at 2nd, 3rd and 4th month after planting in terms of branch number. For plants grown under eCO_2 at different densities, the results were almost the same as those for aCO_2. At first month of growth HDH and HDV produce (11.76) and (11.53) branches per plant while LDH produce (10.13) branches per plant which were significantly lower than HDH and HDV. In the months following until final harvest, no significant difference was recorded for different densities in terms of branch number under eCO_2 treatments (Figure 2b).

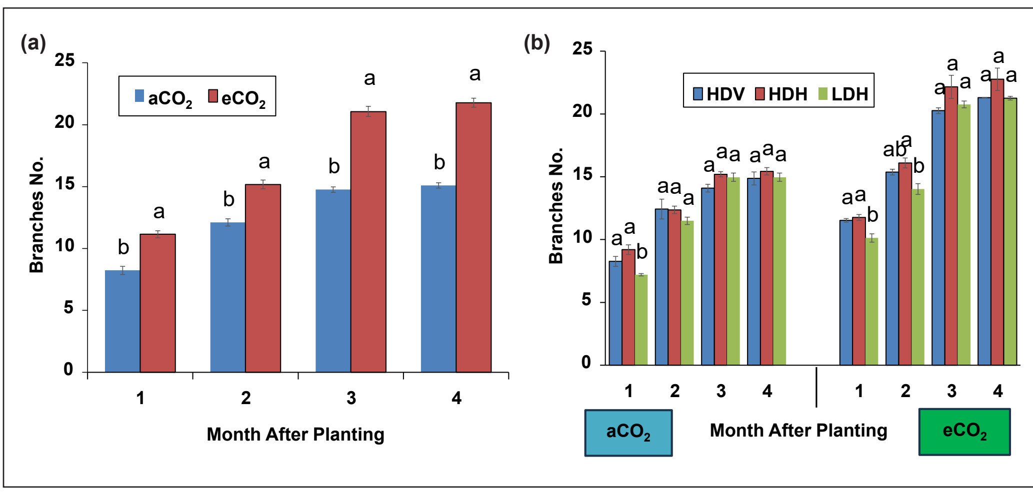

Figure 2. Effect on branching patterns of *Stevia rebaudiana* (Bertoni): (a) CO_2 levels; and (b) planting density
Note. Means with same letter in a group do not show a significant difference according to LSD at $P \leq 0.05$. Error bars indicate the standard error of means

Number of Leaves

The results summarized in Figure 3a show that, similar to other growth parameters such as plant height and branch number, leaf number was also found to be significantly higher under eCO_2 treatment than aCO_2 in normal glasshouse. The highest number of leaves per branch was recorded for plants exposed to eCO_2 throughout the growth period from 1st month of planting until the final harvest at the 4th MAP. In terms of leaf number per branch (18%), increments were seen for CO_2 treated plants at 1st MAP, (17%) in 2nd month, (20%) at 3rd MAP, and (18%) at 4th MAP compared to plants under aCO_2. The findings from the current experiment indicate that plants under different densities with aCO_2 levels do not show any significant difference in the number of leaves in the 1st and 2nd months of growth. At month three the highest number of leaves per branch was recorded for LDH (23.1), followed by HDH (20.03), and HDV (18.93). On the 4th MAP, the LDH plants had the highest number of leaves, and no difference was found between HDH and HDV plants. Under the eCO_2 treatment, the LDH plants produced (13.5), HDV (12.5) and HDH (11.8) leaves per branch. At 2nd month of growth, no significant difference was seen for HDV and LDH plants, whereas HDH produced (14.5) leaves, which were 12% higher than those of HDV and LDH. Similar trends were seen in the 3rd and 4th month as well, where LDH produced a higher number of leaves (Figure 3b).

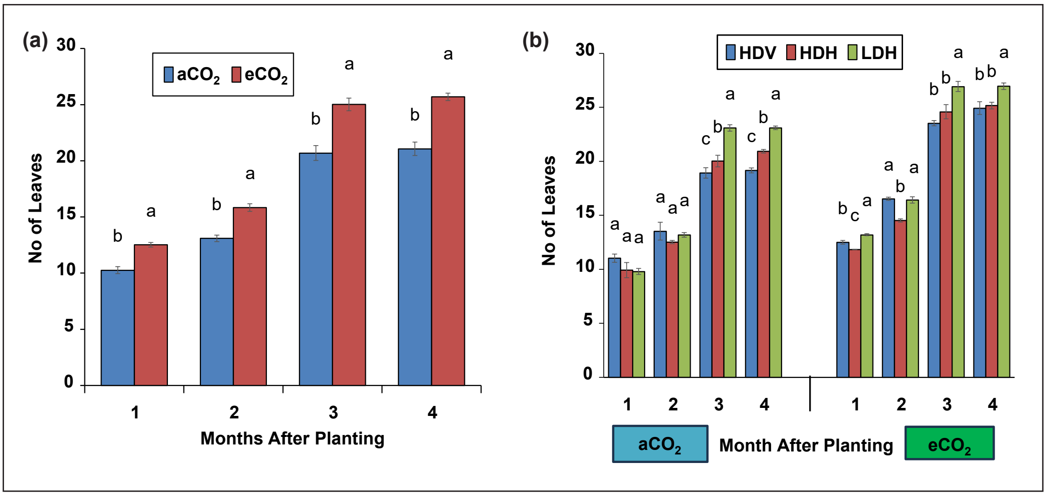

Figure 3. Variation in leaf number of *Stevia rebaudiana* (Bertoni): (a) Under ambient and elevated CO_2; and (b) at different planting densities

Note. Means with same letter in a group do not show a significant difference according to LSD at P≤ 0.05. Error bars indicate the standard error of mean

Photosynthesis (Pn)

Plants exposed to eCO_2 treatment were found to be significantly higher in terms of photosynthetic rates (Pn) compared to plants grown under aCO_2 conditions, regardless of density. On average, Pn was found to be (38%) higher under eCO_2, whereas in the first month, the plants treated with eCO_2 showed (36%) increment while in the 3rd month, the difference was recorded to be (42%) higher than that of plants under aCO_2 concentration (Tables 1 and 2). Our results indicate that plants grown under aCO_2 concentrations with different densities show significant differences in the first month of growth; however, no significant differences were observed in the 3rd month of growth. In the first month of growth, the LDH plants showed the highest values for photosynthesis (12.96 µmol CO_2 m^{-2} s^{-1}), HDV (9.9 µmol CO_2 m^{-2} s^{-1}), and HDH (8.1 µmol CO_2 m^{-2} s^{-1}). On 3rd MAP the higher values were recorded for LDH (6.23 µmol CO_2 m^{-2} s^{-1}) followed by HDH (5.09 µmol CO_2 m^{-2} s^{-1}) and HDV (4.66 µmol CO_2 m^{-2} sec^{-1}) however, the least significant difference test (LSD) at the probability threshold of (≤ 0.05) indicated that the values were not significant. The results summarized in Tables 3 and 4 also show that under the eCO_2 treatment, highly significant differences were observed among plants grown at different densities. The plants grown under LDH were found with highest value of Pn (20.6 µmol CO_2 m^{-2} s^{-1}) followed by HDH (15.3 µmol CO_2 m^{-2} s^{-1}) and

Table 1
Effect of CO_2 levels on leaf gas exchange parameters of Stevia rebaudiana *regardless of planting densities at 1st month of planting*

Parameters	Treatments	
	aCO_2	**eCO_2**
Pn (µmol CO_2 m^{-2} s^{-1})	10.32b ± 0.761	16.20a ± 1.212
gs (µmol CO_2 m^{-2} s^{-1})	0.418a ± 0.045	0.243b ± 0.046
TR (µmol H_2O m^{-2} s^{-1})	2.49a ± 0.095	1.75b ± 0.112
Ci (µmol CO_2 m^{-2} s^{-1})	241b ± 82.44	503a ± 57.33
WUE (µmol CO_2 mol^{-1} H_2O)	4.26b ± 0.450	9.59a ± 0.898

Note. The similar letters given after the number in a column do not show any significant difference according to LSD at 5% probability level. Numbers were followed by ± standard deviation. aCO_2 = Ambient carbon dioxide; eCO_2=Elevated carbon dioxide; HDV=High density vertical; HDH= High density horizontal; LDH=Low density horizontal; Pn=Photosynthetic rate; gs=Stomatal conductance; TR=Transpiration rate; Ci=intercellular CO_2; WUE=Water use efficiency

Table 2
Effect of CO_2 levels on leaf gas exchange parameters to of Stevia rebaudiana *regardless of planting densities at 3rd month of planting*

Parameters	Treatments	
	aCO_2	**eCO_2**
Pn (µmol CO_2 m^{-2} s^{-1})	5.33b ± 0.366	9.33a ± 0.554
gs (µmol CO_2 m^{-2} s^{-1})	0.335a ± 0.027	0.156b ± 0.006
TR (µmol H_2O m^{-2} s^{-1})	5.40a ± 0.097	4.31b ± 0.131
Ci (µmol CO_2 m^{-2} s^{-1})	393b ± 49.18	804a ± 36.58
WUE (µmol CO_2 mol^{-1} H_2O)	0.99b ± 0.077	2.21a ± 0.185

Note. The similar letters given after the number in a column do not show any significant difference according to LSD at 5% probability level. Numbers were followed by ± standard deviation. aCO_2 = Ambient carbon dioxide; eCO_2 = Elevated carbon dioxide; HDV = High density vertical; HDH = High density horizontal; LDH = Low density horizontal; Pn= Photosynthetic rate; gs= Stomatal conductance; TR= Transpiration rate; Ci= intercellular CO_2; WUE= Water use efficiency

Table 3
Effect of CO_2 levels on leaf gas exchange parameters to of Stevia rebaudiana *under different planting densities at 1st month of planting*

Treatments		Parameters				
		Pn (µmol CO_2 m^{-2} s^{-1})	Gs (µmol CO_2 m^{-2} s^{-1})	TR (µmol H_2O m^{-2} s^{-1})	Ci (µmol CO_2 m^{-2} s^{-1})	WUE (µmol CO_2 mol^{-1} H_2O)
aCO_2	HDV	9.9b ± 0.35	0.53a ± 0.03	2.7a ± 0.14	254b ± 43	0.83a ± 0.07
	HDH	8.1c ± 0.06	0.40b ± 0.03	2.5a ± 0.14	201b ± 79	0.93b ± 0.34
	LDH	13.0a ± 0.88	0.312c ± 0.02	2.2a ± 0.03	467a ± 40	1.21b ± 0.08
eCO_2	HDV	12.6c ± 0.58	0.30a ± 0.01	1.90a ± 0.36	430b ± 14	7.31a ± 1.2
	HDH	15.3b ± 0.66	0.29a ± 0.02	1.69a ± 0.04	635a ± 6.9	9.28ab ± 1.3
	LDH	20.6a ± 0.68	0.13b ± 0.03	1.66a ± 0.04	677a ± 14	12.2b ± 1.6

Note. At the 5% probability level, the number in a column that has the same letter is not significantly different by the least significant difference. Thus, numbers were followed by ± standard deviation; aCO_2 = Ambient carbon dioxide; eCO_2 = Elevated carbon dioxide; HDV = High density vertical; HDH = High density horizontal; LDH = Low density horizontal; Pn= Photosynthetic rate; gs= Stomatal conductance; TR= Transpiration rate; Ci= intercellular CO_2; WUE= Water use efficiency

Table 4
Effect of CO_2 levels on leaf gas exchange parameters of Stevia rebaudiana *under different planting densities at 3rd month of planting*

Treatments		Parameters				
		Pn (µmol CO_2 m^{-2} s^{-1})	Gs (µmol CO_2 m^{-2} s^{-1})	TR (µmol H_2O m^{-2} s^{-1})	Ci (µmol CO_2 m^{-2} s^{-1})	WUE (µmol CO_2 mol^{-1} H_2O)
aCO_2	HDV	4.6a ± 0.19	0.40a ± 0.01	5.6a ± 0.21	299b ± 96	3.2a ± 0.8
	HDH	5.1a ± 0.58	0.37a ± 0.00	5.5a ± 0.04	426a ± 32	3.7ab ± 0.6
	LDH	6.2a ± 0.76	0.24b ± 0.04	5.1a ± 0.09	453a ± 56	5.9a ± 1.0
eCO_2	HDV	7.7b ± 0.90	0.17a ± 0.016	4.8a ± 0.079	679b ± 77	1.61b ± 0.4
	HDH	9.3ab ± 0.21	0.15a ± 0.003	4.1b ± 0.049	803a ± 58	2.26ab ± 0.5
	LDH	10.8a ± 0.61	0.15a ± 0.004	4.0b ± 0.093	929a ± 10	2.74a ± 0.8

Note. The number followed by the same letter in a column is not significantly different by the LSD at 5% probability level. Numbers are followed by ± to show standard deviation; aCO_2 = Ambient carbon dioxide; eCO_2 = Elevated carbon dioxide; HDV= High density vertical; HDH= High density horizontal; LDH= Low-density horizontal Pn: Photosynthetic rate gs: Stomatal conductance TR: Transpiration R ate Ci: intercellular CO_2 WUE: Water use efficiency

HDV (12.63 µmol CO_2 m^{-2} s^{-1}) at first MAP. At the 3rd month, the LDH plants were found to be significantly higher than HDV but non-significant to HDH, whereas the HDH plants were not significantly affected by either LDH or HDV in terms of photosynthesis.

Stomatal Conductance (gs)

The results from our current experiment demonstrate that elevated CO_2 has a positive influence on stomatal conductance (gs) of our stevia plant by significantly reducing its frequency. The gs for plants exposed to eCO_2 was found to be reduced by (41%) at the 1st MAP and (53%) at the 3rd MAP compared to plants under aCO_2 treatment, regardless of the density (Tables 1 and 2). Our results shown in Tables 2 and 4 indicate that under aCO_2, the LDH plants showed the lowest gs, followed by HDV, while HDH had the highest values at the 1st MAP. At 3rd month again the lowest values were recorded for LDH, which was significantly lower than HDH and HDV, while no significant difference was observed among plants grown in HDV and HDH. Under eCO_2 conditions, no significant difference was observed for plants grown in HDH and HDV, whereas LDH was found to be significantly lower than both HDV and HDH at 1st MAP. At the 3rd MAP no significant differences were observed between densities.

Transpiration Rate (Tr)

The results of our current study revealed significant alterations in the transpiration dynamics of stevia plants under eCO_2 concentrations. Our results indicate that transpiration rates (Tr) were lower in plants grown under eCO_2 at the 1st MAP and even 3rd MAP than in plants grown under aCO_2 levels (Tables 1 and 2). At the 1st month of growth, the Tr reduction was recorded at (29%) and at the 3rd month of growth, it was (20%) lower than that of plants exposed to aCO_2 level, irrespective of planting density. Plants grown at different planting densities under aCO_2 levels did not show any significant difference in terms of transpiration. The results are summarized in Tables 2 and 4 which illustrated that plants exposed to eCO_2 did not show any significant differences between different planting densities in the first month of growth. However, in the 3rd month, the HDV plants were observed to have the highest Tr values and were found to be significantly higher than HDH and LDH.

Intercellular Carbon Dioxide (Ci)

Intercellular CO_2 (Ci) was recorded for stevia plants under two different CO_2 conditions (aCO_2 and eCO_2) of 400 ppm and 1200 ppm. Measurements were recorded on the 1st and 3rd months of growth. Tables 1 and 2 illustrates that Ci was significantly increased by (52%) in the 1st month of growth and (51%) at 3rd MAP for plants grown under eCO_2 in comparison with plants grown under aCO_2 concentrations, despite the different planting densities. Under aCO_2 concentrations, significant differences were observed for different planting densities. In the first month of growth, the highest value was recorded for LDH plants (466.88 $\mu mol\ CO_2\ m^{-2}\ s^{-1}$), which was significantly higher than HDV (254.73 $\mu mol\ CO^2\ m^{-2}\ s^{-1}$) and HDH (201.35 $\mu mol\ CO_2\ m^{-2}\ s^{-1}$). At 3rd MAP the trend was found to be the same; however, no significant differences were found between LDH and HDH. HDV was found to be significantly lower in terms of Ci than in the LDH and HDH plants. Under eCO_2, the HDV plants were found to have significantly lower Ci than HDH and LDH in the first month; however, the trend was almost the same for all treatments as aCO_2 in the 3rd month of growth (Tables 3 and 4).

Water Use Efficiency (WUE)

The result from the experiment indicates that eCO_2 has positively influenced water use efficiency (WUE) by comparing it with aCO_2 regardless of differences in planting densities. The eCO_2 treated plants observed (55%) increment in WUE in the 1st and 3rd months of growth in plants grown under aCO_2 (Tables 1 and 2). Regarding the planting density, LDH planting showed maximum WUE (5.88 μmol CO_2 mol^{-1} H_2O) followed by HDH (3.19 μmol CO_2 mol^{-1} H_2O) and HDV (3.71 μmol CO_2 mol^{-1} H_2O) under aCO_2 at 1st MAP. The results are summarized in Tables 3 and 4, which shows that at the 1st MAP, the LDH plant showed a significantly higher value for WUE than for HDV. However, no significant difference was observed between HDH and HDV, whereas LDH was not significantly different from HDH. Under eCO_2, the LDH plants showed maximum values for WUE (12.19 μmol CO_2 mol^{-1} H_2O), followed by HDH (9.28 μmol CO_2 mol^{-1} H_2O), which were not significant for HDV (7.30 μmol CO_2 mol^{-1} H_2O) according to LSD at the 5% probability level. The plants showed a similar trend in the 3rd month to the first month (Tables 3 and 4) under eCO_2.

Chlorophyll Content

The results from the current study showed that elevated CO_2 levels positively influenced total chlorophyll content by significantly increasing chlorophyll a and b. Regardless of differences in planting densities, a significant increase in chlorophyll a was observed, which was 25% and 19% higher under eCO_2 treatments than under aCO_2 at the 1st and 3rd MAP respectively (Figure 4a). Under aCO_2 concentration, no significant differences were observed among the different densities at the 1st MAP; however, at the 2nd MAP the HDH plants were found to produce (30.61 µg/mg) non-significant to HDH (28.64) but significantly higher chlorophyll a than HDV (19.95 µg/mg). Significant differences were seen among all three densities under eCO_2 treatment. At 1st MAP no significant differences were observed for LDH and HDV; however, HDH plants significantly higher than LDH and HDV plants in terms of chlorophyll a. At 3rd MAP the HDV plants were seen with significantly lowest values (25.57 µg/mg) than HDH (5.68 µg/mg) and LDH (36.59 µg/mg) for chlorophyll a production (Figure 4b).

Similar to chlorophyll a, eCO_2 concentrations were observed to be increasing chlorophyll b. The highest values (10.72 µg/mg) and (13.96 µg/mg) at 1st and 3rd MAP were recorded for plants grown under eCO_2 level while the plants grown under aCO_2 concentration were seen with significantly lowest values (8.14 µg/mg) and (11.01 µg/mg) for chlorophyll b (Figure 5a). Under aCO_2 concentration no significant difference was observed for all the three densities at 1st as well as 3rd MAP. However, under eCO_2 LDH plants showed significantly higher amount of chlorophyll b than HDV and HDH at both 1st and 3rd month of growth (Figure 5b).

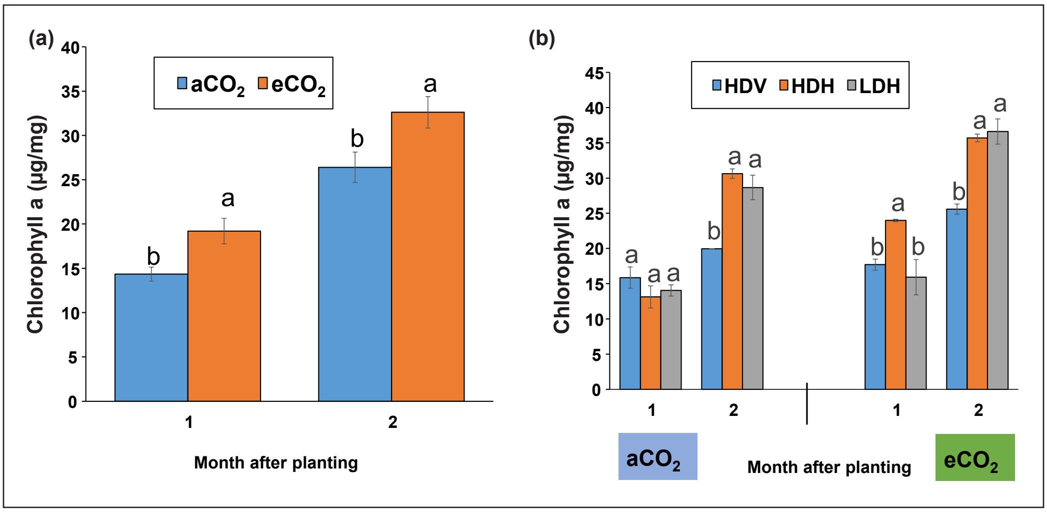

Figure 4. Variation in chlorophyll a concentration: (a) Under ambient and elevated CO_2; and (b) at different planting densities

Note. Means with same letter in a group do not show a significant difference according to LSD at P≤ 0.05. Error bars indicate the standard error of mean

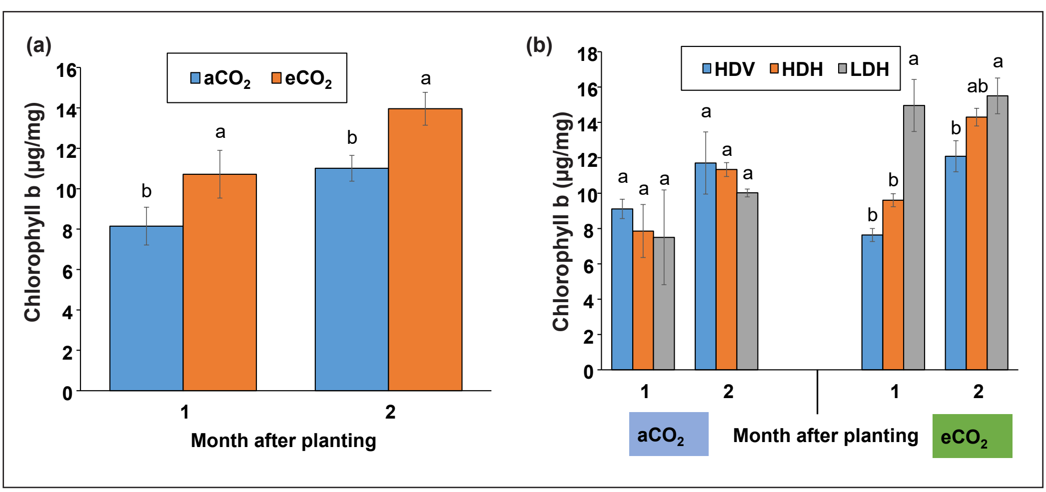

Figure 5. Variation in chlorophyll b levels: (a) Under ambient and elevated CO_2; (b) at different planting densities

Note. Means with same letter in a group do not show a significant difference according to LSD at P≤ 0.05. Error bars indicate the standard error of mean

The maximum total chlorophyll was recorded for eCO_2 treated plants at 3^{rd} MAP (46.91 µg/mg) and (29.92 µg/mg) at 1^{st} MAP while the aCO_2 plants produced (37.22 µg/mg) at 3^{rd} MAP and (22.49 µg/mg) of total chlorophyll at 1^{st} MAP which were significantly lower than eCO_2 treatment (Figure 6a). The plant exposed to aCO_2 with different planting densities does not show any significant difference in the 1^{st} month of growth while at 3^{rd}

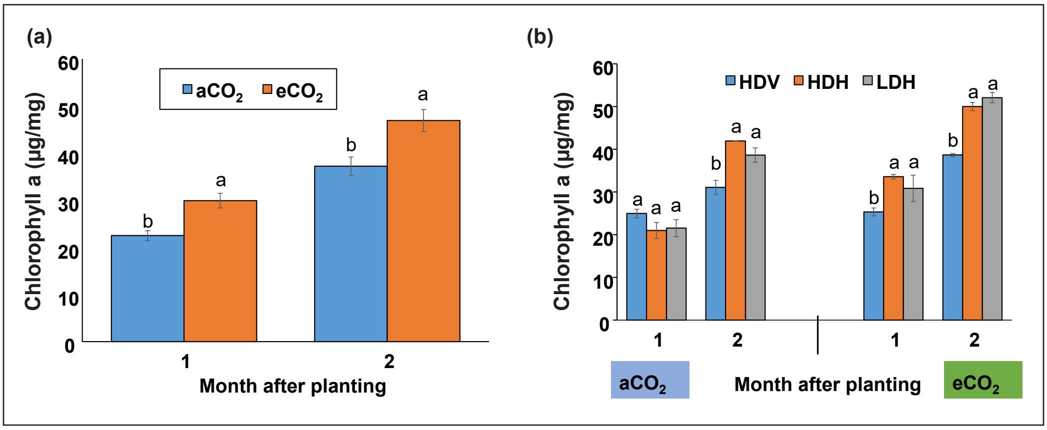

Figure 6. Impact on total chlorophyll content of *Stevia rebaudiana* (Bertoni): (a) CO_2 levels; and (b) plant density
Note. Means with same letter in a group do not show a significant difference according to LSD at P≤ 0.05. Error bars indicate the standard error of mean

month of growth the LDH and HDH plants were observed with significantly higher total chlorophyll than HDV. Under eCO_2 no significant was observed between HDH and LDH at 1st and 3rd MAP. However, HDV was seen with significantly lowest total chlorophyll at 1st as well as 3rd MAP (Figure 6b).

DISCUSSION

The results of the current experiment show that plant growth parameters specifically, plant height, branch number, and number of leaf were positively affected by elevated CO_2 (eCO_2) treatment compared to ambient CO_2 (aCO_2) in *Stevia rebaudiana*. As shown in Figures 1, 2, and 3, the eCO_2 treated plants were observed with maximum values for plant height, branch number, and leaf number throughout the growth period, from the first month after planting (MAP) until the final harvest at the fourth MAP. Increase in photosynthetic rates is the primary mechanism that promotes plant growth. This process also helps plants undergo morphological changes, such as changes in the ratio of roots to shoots, an increase in number of leaves and the production of tillers (Salihi et al., 2023). It is a common knowledge and has been revealed by various scientific reports that eCO_2 enrichment boosts photosynthesis in most of the C_3 crop like stevia by increasing the rate of carboxylation reaction and reduces oxygenation, hence producing more sugar, leading to an increase in plant growth. CO_2 reduces oxygenation reaction which alternately prevents the production of glycolate due to which plant saves more energy and spends those extra ATP, s for plant growth (Zhang et al., 2013; Zhu et al., 2010). An increase in height may be because of the fast growth of *Stevia rebaudiana* under CO_2 enrichment which is results of increase in cell division and elongation as Maity et al. (2019) reported similar results for rice plant height from 76.9 cm under aCO_2 to

81.7 cm with eCO_2 exposure. It has been reported by Seneweera et al. (2011) that 50% increment was seen for Jarrah rice variety under eCO_2 (700 ppm) which support our current results for increased in branches number, as Stevia is C_3 crop and most of the C_3 crop showing similar response to environmental factors like CO_2. Our results in term of leaves show similarity to Costa et al. (2003) where they reported that leaf number and LAI were significantly higher under eCO_2 (570 ppm) than ambient CO_2 (370 ppm). Our results are also in agreement with those of Saha et al. (2015) who observed that height for chickpea plants increased when exposed to eCO_2 concentration. Thilakarathna et al. (2015) also suggested that under eCO_2 level there will be high carbon supply to plant cells which may accelerate its division and expansion in meristematic tissues which enhance growth and development of the plant. Additionally, eCO_2 by positively enhancing the expression of genes which is responsible for growth, cell division and cell wall properties in plants. For example, eCO_2 up regulates genes that encode for expansins, xyloglucan, endotransglucosylase and pectin esterase which play an essential role in cell wall loosing and ultimately helping leaf expansion in plants (Desouza et al., 2008; Wei et al., 2013). Likewise, the ribosomal protein genes that control the cytoplasmic development of plant cells and cell cycle genes encoding cyclin, cyclin-dependent protein kinase, tubulin, and cyclin-dependent protein kinase regulator were shown to be up-regulated at eCO_2 level (Ainsworth et al., 2006; Wei et al., 2013). Plant cells under eCO_2 may divide, develop, and expand more often due to the up regulation of these enzymes involved in the cell cycle and cytoplasmic proliferation which contribute to maximizing growth and development of the plant.

Besides the enhancement in photosynthesis and gene expression under elevated carbon dioxide, it has also been reported that plant hormones are playing an essential role in modification of plant growth and development (Desouza et al., 2008; Teng et al., 2006; Wei et al., 2013). Some of the fundamental plant's hormones are auxins, gibberellic acids and cytokinin's that are mutually stimulating and regulate the division and expansion of the cells as well as control shoot meristem development and stem elongation (Cato et al., 2013; Gamage et al., 2018). Teng et al. (2006) used *Arabidopsis thaliana* to study the effects of eCO_2 on plant hormones and reported that there was a significant increase in gibberellic acid, zeatin riboside, dihydrozeatin riboside, isopentenyl adenosine of the class cytokinin, and indole-3-acetic acid, one of the common plant hormones in the auxin class. It has also been suggested by several other studies that eCO_2 significantly increases transcript abundance of genes that are related to synthesis and transport of auxins, gibberellic acid and cytokinin (Desouza et al., 2008; Gamage et al., 2018; Wei et al., 2013). All of these findings may explain why all the growth-related parameters like plant height; number of branches and leaves were significantly higher under eCO_2 concentration in comparison to aCO_2 for *Stevia rebaudiana* in the current experiment.

Different planting densities did not show any significant effects on plant height and branch development, specifically at the later growth stages under ambient CO_2 (aCO_2). However, at the first month after planting (MAP), plants grown under high-density vertical farming (HDV) showed reduced plant height, and at the second month, high-density horizontal planting (HDH) plants showed significantly lower plant height compared to low-density horizontal planting (LDH) and HDV. Under elevated CO_2 (eCO_2) concentration, only HDH plants exhibited significantly lower plant height at both the first and second MAP. No significant differences were observed in plant height across all planting densities at the third and fourth MAP under eCO_2. Results from current experiments are being supported by Benhmimou et al. (2017) where they observed that narrow spacing with plant populations of 142857 plants ha^{-1} resulted low plant height by comparing with wider spacing with a plant population of 47619 plants ha^{-1}. However, results from eCO_2 enrichment showed higher plant height in HDV plants which are supported by Mahajan and Pal (2021) by reporting that plant height was improved with dense planting. Similarly, the number of branches were observed with significant difference at 1st MAP only where the LDH plant produce significantly lower branches than HDH and HDV under both aCO_2 and eCO_2 while no significant were found at 2nd , 3rd and 4th MAP respectively. The higher plant height at early growth stages under high plant density could be due to the receiving of less radiation at ground/basal portion, therefore elongation of bottom internodes increases. Under dense canopy the light received is poor in red radiations and enriched with far-red radiation which strengthens apical dominance by stimulating internodes elongation (Rajcan & Swanton, 2001; Xue et al., 2016). Ozer (2003) reported that plant height in *Brassica napus* was higher under high plant density and similarly, higher plant height was also documented for cotton (*Gossypium hirsutum* L.) by several authors under high density planting (Clawson et al., 2006; Gwathmey & Clement, 2010; Khan et al., 2020). In this study, for high density planting, the number of branches were higher under eCO_2 at 1st and 2nd MAP which could be possible due to CO_2 enrichment as the plants were not suffering from resource limitations, specifically plant nutrition. Leaf is an important and economical part of stevia rebaudiana as steviol glycosides are present in higher amount in leaves than other parts of the plant. Under aCO_2 significant difference were seen at late growth stages where the LDH plants produce higher leaves followed by HDH and HDV produce significantly lower leaves. Similarly, the leaves number were higher for low density planting under eCO_2 conditions. Under low density planting, the competition for available resources is less due to less inter-row and intra-row spacing hence there is higher chance for plant to develop greater number of leaves than high density planting. The results for higher leaves number under LDH show similarities to Kumar et al. (2014) and Tadesse et al. (2016) where they reported that wider spacing produced significantly higher number of leaves per plant in comparison to narrow spacing. However, the high number of leaves per unit area can be achieved from high density planting which is due to the increasing number of plants per unit area.

Results from the current study showed that leaf gas exchange parameters were positively influenced by eCO_2 despite the differences in planting densities. Photosynthetic rate (Pn), intercellular CO_2 (Ci) and water use efficiency (WUE) were significantly increased under eCO_2 while transpiration rate (Tr) and stomatal conductance (gs) decreased with increasing the concentration of CO_2. Carbon dioxide shows the first and most direct effect on photosynthesis of the plants. Because CO_2 is the raw material in the process of photosynthesis that is significantly affecting production of the plant (Zhang et al., 2013). There are three main phases in light independent reaction of photosynthesis which are carboxylation, reduction and regeneration. The Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) enzyme is responsible for fixing CO_2 to ribulose-1,5-bisphosphate (RuBP), resulting in a 3-phosphoglycerate (PGA) molecule (Dusenge et al., 2019). Due to the low affinity of Rubisco to CO_2 it can bind to O_2 and lead to photorespiration producing glycolate, as the carboxylation reaction of RuBP is not saturated at the current atmospheric CO_2. Therefore, in the C_3 crop, eCO_2 induced photosynthesis to increase in two different ways. First, improves carboxylation level, Second, CO_2 prevented the oxygenation/photorespiration reaction and reduces glycolate production which is wasteful in terms of energy, as it costs the plant more energy and does not lead to any gains in energy or carbon (Dusenge et al., 2019; Peterhansel et al., 2010). The similar results to our findings for high Pn under eCO_2 were also reported by Ji et al. (2015). Our results also show similarity to Javaid et al. (2022) where they reported that Pn was increased by 25% with eCO_2 by comparing to that of aCO_2 concentration. Additionally, it is commonly believed that CO_2 enrichment increases photosynthesis in plants, even under stressful environments (Dekauwe et al., 2021; Ghahramani et al., 2019). According to Park and Runkle (2018), the Pn rate was increased under eCO_2 concentration which supports our current results.

Stomatal conductance and transpiration rate were found to be inversely proportional to photosynthesis with increase in CO_2 concentration. The plants under eCO_2 were observed with significantly lower values for gs and Tr than aCO_2 treated plants. The similar findings are reported by Yasutake et al. (2016) where they observed that the Pn was significantly increased while gs and Tr were decreased under eCO_2 concentration. The low gs could be the reason for the low Tr under CO_2 enrichment. Our results for gs and Tr are in line with Ahmed et al. (2022) where the reported low gs and Tr for Lettuce plants under elevated CO_2 concentration of (1000 & 1500 ppm) in comparison to (500 ppm). The similar results to our findings for reduction in Tr and gs were also reported by Lamichaney et al. (2021) under eCO_2 (566-630 ppm) in comparison to (379−423 ppm) for chickpea plants. Our results are also in line with Wang et al. (2018) who reported the decline of Tr and gs under CO_2 enrichment. The Ci was observed with a significant increment in response to

CO_2 enrichment. However, in the 3rd month of growth the Ci concentration was higher as compared to the 1st MAP. The reduction of Ci was related to photosynthesis. As mentioned in earlier section that a high Pn was recorded under eCO_2 at 1st MAP than 3rd MAP. When the plant performs high photosynthesis the concentration of CO_2 in leaves decreases because CO_2 is an essential element that is used in the process of photosynthesis. The results from our experiment are in line with Fathurrahman (2023) where he reported higher Ci with eCO_2 (800 ppm). The similar results to our finding are also reported by Javaid et al. (2022) with Ci enhancement under eCO_2 concentration. However, in contrast to our findings, Lamichaney et al. (2021) reported a decline in Ci under eCO_2 and explain that under eCO_2 conditions the reduction in Ci could be due to greater affinity of CO_2 binding with Rubisco enzyme leading to higher photosynthesis. The results for the current experiment showed significantly higher WUE upon the exposure of plants to eCO_2 concentration especially at early growth stages. The higher WUE may be due to low transpiration rate as results of low gs under eCO_2 treatment. In fact, it is clearly understood from our results along with finding from other researches that enhancement in WUE is the result due to higher Pn with low gs under elevated carbon dioxide concentration (Wei et al., 2022). Our finding is supported by Javaid et al. (2022) where they reported a 40% higher WUE for *Datura stramonium* under eCO_2 conditions in comparison to aCO_2. Similarly, another study by Walia et al. (2022) reported that eCO_2 increased WUE by decreasing Tr and gs due to partial closure of stomata. Our results are also in line with Robredo et al. (2007) where they found increased WUE for barely plants grown under eCO_2 than those grown under aCO_2 conditions.

Leaf gas exchange plays a central role in biomass and yield accumulation as these traits are the base of plant physiology. The Pn which reflects photosynthetic capacity and potential productivity is one of the most important physiological trait (Xia et al., 2020). In the current study Pn was significantly higher for LDH while HDV and HDH do not show any significant at 1st MAP however, at 3rd MAP all the three densities were non-significant under aCO_2. The similar results were seen for all densities under eCO_2. The low Pn could be due to the low quality of light intensity with increased canopy layer under high planting density and gradual development of shading due to increasing number of *Stevia rebaudiana* leaves under high planting density. The similar results are reported by Huang et al. (2021) with high Pn under low planting density for *Cunninghamia lanceolata*. Our results are also in agreement with Mwamlima et al. (2020) where they reported significantly higher Pn under low planting density for Soybean. Significantly higher Ci and WUE were recorded for low planting density. However, the Ci was higher at maturity while WUE was maximum at early growth stages. This might be due to high photosynthesis performance at early growth stages where the plant uses more CO_2 and water. On the other hand, at maturity low photosynthesis might be due to the lack of assimilating photosynthetic material which decreases WUE and increases CO_2 at the cellular level (Tang et al., 2015). The similar results for high WUE and Ci were reported by Huang et al. (2021) which are supporting our findings for the current experiment. The result from the current study showed maximum gs for HDV than LDH and HDH under aCO_2 while under eCO_2 the trend was similar to aCO_2 at 1st MAP but no significant difference was seen at 3rd MAP between all the three densities. In term of Tr the HDV was found to be significantly higher than HDH and LDH under eCO_2 at 3rd MAP while no significant were seen for aCO_2 between HDV, HDH and LDH. Our results for low gs from the current study agree with Koesmaryono et al. (1997), Zhou et al. (2011) and Moreira et al. (2015) where all of them reported that under low planting density the stomatal conductance decreased. Similarly, the non-significant results for Tr agree with Wilson et al. (2012) where they reported that varying planting density does not affect Tr.

Chlorophyll is an important pigment which is found in the chloroplast of the plant and plays a central role in the processes of photosynthesis, that's why it is very important for plant growth and yield (Ohmiya et al., 2017). Results from the current study showed that chlorophyll a, b and total chlorophyll were significantly increased under elevated CO_2. Our results for high photosynthesis in the earlier section might explain the increment in chlorophyll under CO_2 enrichment. Results from current study are consistent with previous finding from Song et al. (2020) who proved that CO_2 enrichment has positively enhanced photosynthesis by increasing chlorophyll content in cucumber. In order to find the reason for enhanced photosynthesis and chlorophyll increment under eCO_2, Song et al. (2020) screened 17 genes related to chlorophyll by transcriptome sequencing and found that most of the genes were upregulated under CO_2 enrichment which may explain the increase in chlorophyll under eCO_2 for our stevia plant as well. Our results are also supported by Samuolienė et al. (2006) where they reported that eCO_2 (1500 ppm) significantly increased chlorophyll content in radish. Results from our study are also similar to Yang et al. (2023) where they mention 7.6% increment in chlorophyll content under eCO_2. Naznin et al. (2015) reported increment in chlorophyll content in garlic under eCO_2 level which are supporting our current results for increased chlorophyll content in stevia. Regarding the differences in planting densities, no significant difference was found under aCO2 for chlorophyll a and total chlorophyll at first MAP. However, at 3rd MAP HDV were seen with low significant value than HDH and LDH. Overall chlorophyll b was found to be non-significant for all three densities. under eCO_2 HDV were found with significantly lower values of chlorophyll a and total than LDH and HDH at 1st as well as 3rd MAP. Under high density planting the number of leaves increased per unit area which cause shading lower down plant capacity to capture light. The minimizing of light capturing decreases the carboxylation capacity and mesophyll conductance which lead to low concentration of chlorophyll content per unit area of leaf. For the majority of plant species, reduced numbers of chloroplasts and lower fresh mass per area offset higher levels of chlorophyll per unit of fresh mass and per chloroplast in shaded leaves (Lambers et al. 2008). Our current results are in line with Gomes et al. (2018) where they reported a decrease in chlorophyll content under high plant density for Stevia rebaudiana. Similarly, Zhang et al. (2021) reported that chlorophyll content in maize leaves were respectively decreased under high planting density compared to low planting densities. On the other hand, Mahajan and Pal (2021) reported that overall, no significant effect was found in chlorophyll content under high and low planting density. However, chlorophyll content was slightly decreased with dense planting which are in support of our results.

CONCLUSION

The results from the current experiment show that elevated eCO_2 boosts the growth of *Stevia rebaudiana*, positively enhancing plant height, branch number, and leaf production, basically due to increased photosynthesis and water use efficiency. Additionally higher chlorophyll content supports the enhancement in growth pattern. While lower planting density promotes leaf production by reducing competition, eCO_2 improves growth across all densities. These results suggest that eCO_2 enrichment can optimize Stevia cultivation, enhance yield and plant productivity in horizontal farming planting setups rather than vertical farming.

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Assessing Reproductive Ecology and Oviposition Habitat Selection Among Anurans in Ayer Hitam Forest Reserve, Puchong, Selangor, Malaysia

Nor Azman Izzah Nadzirah[1], Mohd. Top @ Mohd. Tah Marina[1,2]* and Wan Mukadza Wan Nurul Nazirah[1]

[1]Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Centre of Foundation Studies in Science of Universiti Putra Malaysia, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

This study explored the breeding ecology and oviposition site selection of anurans in Compartment 15 of Ayer Hitam Forest Reserve (AHFR), focusing on the relationship between habitat variables and oviposition sites. The effectiveness of artificial breeding ponds was also assessed. Ten breeding ponds (five natural, five artificial) were studied from June to November 2022. Frog sampling occurred twice weekly at night, and tadpoles were observed weekly. Microclimate, macrohabitat data, and anuran presence were recorded. A total of 67 anurans from 18 species and six families were documented. *Hylarana labialis* had the highest number of individuals (12) near natural ponds, while *Kalophrynus palmatissimus* had the same number across natural and artificial ponds. The latter species, endemic to the region, is classified as Endangered (EN) by the IUCN Red List Index 2024. Tadpoles of *H. labialis*, *K. palmatissimus*, and *Microhyla* sp. were also documented. The study provides valuable insights into anuran habitat selection and microclimate influences. While these species show adaptability in disturbed areas, further research is needed to understand the impact of forest disturbance on their breeding ecology, as habitat loss could affect their populations.

Keywords: Artificial breeding ponds, conservation, endangered (EN) species, microclimate, macrohabitat

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E-mail addresses:
izzah1206@gmail.com (Nor Azman Izzah Nadzirah)
marinamohd@upm.edu.my (Mohd. Top @ Mohd. Tah Marina)
wannurulnaz5277@gmail.com (Wan Mukadza Wan Nurul Nazirah)
* Corresponding author

INTRODUCTION

The success of anuran reproduction is heavily influenced by the selection of suitable breeding sites (Buxton et al., 2016), which directly impacts larval survival, development, and overall population dynamics (Rudolf & Rödel, 2005). Numerous studies have explored the habitats and ecology of anuran

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species; however, a significant gap remains in our understanding of artificial breeding ponds in Malaysia. This study seeks to address this gap by focusing on the conservation of endangered anuran species within the Ayer Hitam Forest Reserve (AHFR) in Puchong, Selangor. Specifically, we aim to provide insights into the breeding ecology and oviposition site selection of anurans, with a particular emphasis on endangered species in AHFR.

The selection of oviposition sites has a direct impact on biological fitness and is influenced by numerous environmental factors, which include predator presence, water temperature, risk of desiccation, the substrate for laying eggs, and the chemical characteristics of the water body (Sánchez-Ochoa et al., 2020). Oviposition site selection in anurans is influenced by abiotic and biotic factors, with parents often choosing sheltered locations that retain moisture and provide cover to protect eggs from desiccation and predation, while factors like nest moisture, pH, proximity to water, and predator presence play key roles in determining site quality (Fischer, 2023). The relative importance of a single factor might depend on the impact of other habitat parameters, making the selection of oviposition sites context-dependent (Fischer, 2023; Reich & Downes, 2003). Additionally, tropical rainforests are highly dynamic ecosystems where the availability and quality of suitable breeding habitats can fluctuate unpredictably (Peigner et al., 2023). In regions like AHFR, where natural water bodies may be limited or degraded, artificial ponds could serve as essential breeding habitats for amphibians, thereby playing a crucial role in their conservation (Brand et al., 2010).

Few studies consider multiple selective forces on oviposition site selection, which limits our understanding of the relative impact of different processes on this critical life history trait and hinders the development of effective conservation strategies for frogs (Sánchez-Ochoa et al., 2020). To address this, our research investigates the factors influencing anuran oviposition site selection within AHFR, focusing on both natural and artificial breeding sites. This involves documenting the anuran species present near these breeding ponds and assessing the effectiveness of artificial ponds in supporting anuran reproduction. By identifying key microclimate and macroclimate parameters that influence habitat selection, this study will contribute to a better understanding of how artificial breeding ponds can support anuran populations, particularly those that are endangered.

Amphibians are among the most threatened vertebrates, facing significant extinction risks due to habitat loss, diseases, and over-exploitation (Bates et al., 2019; Luedtke et al., 2023; Whittaker et al., 2013). The Global Amphibian Assessment (GAA2) on the IUCN Red List highlights that 41% of amphibian species are experiencing enigmatic declines, with habitat loss being a primary concern, especially in agricultural and urbanized regions (Luedtke et al., 2023). In such areas, artificial ponds may serve as the only viable breeding habitats for amphibians, underscoring their potential importance in conservation efforts (Brand et al., 2010).

Thus, while we have a good understanding of how ovipositing organisms respond to single factors, we still know little about how these responses translate into more complex situations with multiple factors. Identifying the factors influencing oviposition site selection in amphibians is crucial for the conservation of threatened, geographically restricted, and rare species. Thus, while we have a good understanding of how ovipositing organisms respond to single factors, we still know little about how these responses translate into more complex situations with multiple factors. Identifying the factors influencing oviposition site selection in amphibians is crucial for the conservation of threatened, geographically restricted, and rare species. This study seeks to address the question: What are the factors influencing oviposition site selection in anurans, and how effective are artificial breeding ponds in supporting anuran reproduction within AHFR? The findings will not only inform future research but also guide conservation strategies aimed at preserving amphibian populations in the tropical forest of Malaysia and similar ecosystems worldwide.

METHODS

Study Area and Sampling Sites

In this study, sampling was conducted in Compartment 15 of AHFR, Puchong, Selangor, focusing on two types of breeding ponds: natural and artificial. The geographic coordinates of each site were recorded using a Garmin GPSMAP 64S Handheld device. Environmental data were systematically documented, including the geographic location of each pond, the density of surrounding vegetation, light intensity, ambient temperature, microhabitat structures within the ponds, and the dimensions (size and depth) of the ponds.

For artificial breeding sites, black trays measuring 98 cm × 67 cm × 9 cm were utilized as experimental ponds. Initially, these trays contained only rainwater, and no substrate (e.g., leaves, sand, or stones) was introduced manually; instead, substrates accumulated naturally over time.

Anuran Sampling and Environmental Monitoring

Anuran populations were sampled within a 20-meter radius of the breeding ponds across all 10 sites. The sampling frequency was increased to twice a week (Friday and Saturday nights) from July 2022 to November 2022, as opposed to the once-every-three-weeks schedule used in previous studies (e.g., Caballero-Díaz et al., 2022). Sampling was conducted in diverse habitats, including swampy areas, trails, and stream edges. Captured anurans were temporarily contained in ventilated spherical plastic containers (Hidayu, 2019; Nadia et al., 2020).

During sampling, various microhabitat structures and environmental parameters were recorded. Microclimate data, including environmental temperature (°C), light intensity (lux), wind speed (mph), and humidity (%), were monitored using a Lutron-LM-8010 anemometer (Nadia et al., 2020). Soil pH was measured with a Takemura DM-15 pH soil instrument (Faris, 2019), while water parameters, including temperature (°C), pH, and dissolved oxygen levels (mg/L), were assessed using a Myron L UltraPen PT2 pH meter and a BLE-9100 dissolved oxygen analyser, respectively.

Data Collection and Analysis

Comprehensive data collection included measurements of egg clutches, egg sizes, and larval stages within the breeding ponds. Microorganisms present in the ponds were collected and examined under an Olympus microscope in a laboratory setting. Adult anurans encountered in the sampling areas were captured for sex determination and body measurements, which included weight, snout-vent length (SVL), head length (HL), head width (HW), snout length (SL), tibia length, and hindlimb length, all measured with a vernier caliper (Gvoždík et al., 2008; Nadia et al., 2020).

Captured anurans were transported to the AHFR laboratory for species identification. High-resolution photographs of each specimen were taken from dorsal, lateral, and ventral perspectives. Identification was conducted using Norhayati Ahmad's (2017) reference book 'Frogs and Toads of Malaysia' and the online database 'Ecology Asia' (www.ecologyasia.com). To prevent repeated sampling of the same individuals, each anuran's foot was marked with non-toxic nail polish before they were released back into their original habitat.

Statistical Analysis

Species diversity was quantified using the Shannon-Weiner diversity index (H), which accounts for both species richness and the relative abundance of each species within the community (Kassie et al., 2023). Species evenness, representing the uniformity of species abundances, was calculated using Pielou's evenness index. Data were analysed using Paleontological Statistics (PAST) software Version 3.18. The Shannon-Weiner Diversity Index was calculated using Equation 1:

$$\boldsymbol{H'} = -\sum_{j=1}^{S} \rho_i \ln \rho_i$$

$$\boldsymbol{E} = \mathrm{H}/\ln(\mathrm{S}) \quad [1]$$

Where ρ_i = proportional abundance, H' = Diversity Index, E = evenness index, and S = the total number of species

All data were recorded in Microsoft Excel and analysed using IBM SPSS Statistics 27. An independent t-test was employed to assess significant differences between natural and artificial breeding ponds in terms of microclimate (Kim, 2015). Pearson's correlation coefficient (r) was utilized to examine relationships between oviposition site selection and various habitat variables (Hazra & Gogtay, 2016; Yadav, 2018). Additionally, a Kruskal-

Wallis test was applied for overall comparisons between anuran species and study areas (McKight & Najab, 2010), and a Spearman's Correlation Coefficient test was conducted to explore correlations between anuran species and microclimate variables (Zar, 2005). This approach ensures rigorous data collection and analysis, facilitating a comprehensive understanding of anuran diversity and the factors influencing their breeding ecology.

RESULTS AND DISCUSSION

Anuran Species Recorded in Ayer Hitam Forest Reserve, Puchong

Sampling within a 20-meter radius of each breeding pond, including nearby trails, streams, and swampy terrain, in Compartment 15 of AHFR, Selangor, recorded 67 individuals from 18 species across six families (Table 1). Newly recorded species included *Kurixalus chaseni* and *Nyctixalus pictus*. According to Hazieq (2023), AHFR harbors approximately 39% of the region's amphibian species, identifying 43 species from six families from 1975 to 2023. Since 2019, three newly recorded species have been documented, including the two from this study.

Table 1
The snout-vent length and sex of anuran records

No	Family	Species	SVL range (mm)	SVL mean ± SD/SE (mm)	Sex	
					M	F
1.	**Bufonidae**	*Duttaphrynus melanostictus*	73.0	73.0	1	0
2.		*Ingerophrynus quadriporcatus*	31.0	31.0	1	0
3.	**Dicroglossidae**	*Fejervarya cancrivora*	35.0 –73.0	57.7 ± 18.1/9.1	1	2
4.		*Limnonectes blythii*	30.0 – 103.0	66.5 ± 51.6/36.5	1	1
5.		*Limnonectes malesianus*	35.0 – 102.0	55.3 ± 23.9/9.0	4	2
6.		*Occidozyga laevis*	24.0 – 39.0	33.5 ± 6.7/2.7	6	1
7.	**Megophrynidae**	*Leptobrachium nigrops*	45.0	45.0 ± 0.0	0	2
8.	**Microhylidae**	*Kalophrynus palmatissimus*	34.0 – 43.0	38.1 ± 3.5/1.0	8	4
9.		*Microhyla berdmorei*	21.0 – 28.0	24.5 ± 4.9/3.5	1	1
10.		*Microhyla mantheyi*	26.0	26.0	1	0
11.	**Ranidae**	*Hylarana glandulosa*	36 – 92	69.5 ± 23.7/11.9	0	4
12.		*Hylarana labialis*	16.0 – 48.0	35.8 ± 10.6/4.3	7	5
13.		*Hylarana laterimaculata*	43.0	43.0	0	1
14.		*Hylarana nicobariensis*	37.0 – 45.0	41.8 ± 4.0/1.8	2	3
15.	**Rhacophoridae**	*Kurixalus chaseni*	34.0 – 36.0	35.0 ± 1.4/1.0	1	2
16.		*Nyctixalus pictus*	35.0	35.0	0	1
17.		*Polypedates leucomystax*	43.0	43.0	0	1
18.		*Rhacophorus pardalis*	45.0 – 57.0	53.0 ± 6.9/4.0	2	1
Total			-	-	**36**	**31**
Total no. of individuals				**67**		

Note. SVL = snout-vent length; F = female; M = male

The relatively low number of anuran individuals recorded in this study (67 individuals) may be attributed to several ecological and methodological factors. Amphibian populations are highly influenced by seasonal variations and climatic conditions, particularly rainfall and temperature, which affect breeding activity and calling behaviour (Mehra et al., 2021).

Ayer Hitam Forest Reserve (AHFR) has undergone significant habitat modification due to historical logging and ongoing anthropogenic activities. Compartment 15, in particular, is situated near a developed area featuring visitor facilities and a gravel road and has been designated as a development and demonstration zone (Abdullah et al., 1999; Sa'adah, 2018). Such modifications can reduce habitat connectivity, alter microclimatic conditions, and negatively impact anuran populations, particularly ground-dwelling species (Püttker et al., 2020; Ramalho et al., 2022). Comparatively, Faris (2016) also recorded a low number of 45 individuals from the same compartment.

Of the total, 51 individuals from 16 species across six families were recorded at natural ponds, while artificial ponds hosted 16 individuals from nine species across four families (Table 2). Natural ponds had the highest abundance of Ranidae (19), followed by Dicroglossidae (13) and Microhylidae (10). In artificial ponds, Dicroglossidae and

Table 2
Number and species of anurans in two types of breeding ponds

No	Family	Species	No. of Individuals (n)		
			Natural Pond	Artificial Pond	Total
1.	**Bufonidae**	*Duttaphrynus melanostictus*	0	1	1
2.		*Ingerophrynus quadriporcatus*	1	0	1
3.	**Dicroglossidae**	*Fejervarya cancrivora*	2	1	3
4.		*Limnonectes blythii*	1	1	2
5.		*Limnonectes malesianus*	3	3	6
6.		*Occidozyga laevis*	5	2	7
7.	**Megophrynidae**	*Leptobrachium nigrops*	2	0	2
8.	**Microhylidae**	*Kalophrynus palmatissimus*	7	5	12
9.		*Microhyla berdmorei*	2	0	2
10.		*Microhyla mantheyi*	1	0	1
11.	**Ranidae**	*Hylarana glandulosa*	3	1	4
12.		*Hylarana labialis*	12	0	12
13.		*Hylarana laterimaculata*	0	1	1
14.		*Hylarana nicobariensis*	4	1	5
15.	**Rhacophoridae**	*Kurixalus chaseni*	3	0	3
16.		*Nyctixalus pictus*	1	0	1
17.		*Polypedates leucomystax*	1	0	1
18.		*Rhacophorus pardalis*	3	0	3
Total no. of individual			**51**	**16**	**67**
Total species (%)			**16 (89%)**	**9 (50%)**	**18**

Microhylidae were the most abundant (five each). Certain species, including *Ingerophrynus quadriporcatus*, *Leptobrachium nigrops*, and *Nyctixalus pictus*, were exclusive to natural ponds, while *Duttaphrynus melanostictus* and *Hylarana laterimaculata* were unique to artificial ponds.

Hylarana labialis had the highest number of individuals (12), predominantly found on Bertam tree leaves near natural ponds. Its absence near artificial ponds might be due to its location in open areas without nearby vegetation. This species, tolerant of habitat disturbances, is associated with forested streams, as reported by Bahiah et al. (2019) and the IUCN (2024). Similar findings have documented its presence on palm leaves, such as *Pandanus* and *Licuala grandis* (Inger & Stuebing, 2005).

Kalophrynus palmatissimus, another abundant species (12 individuals), was recorded across five ponds, including natural ponds (NP II, NP III) and artificial ponds (AP I, AP III, AP IV). Consistent with previous studies (Faris, 2019; Faris et al., 2021; Hidayu, 2019; Nadia, 2017; Norhayati, 2017; Sa'adah, 2018), this species prefers forest litter and open areas like trekking trails. Similarly, Badli-Sham (2023) reported its presence in open areas, such as camping sites and trails, at Sekayu Recreational Forest in Terengganu. Additionally, *Kalophrynus* species utilize temporary ponds and phytotelmata (e.g., root buttresses and hollow tree trunks) for breeding (Haas et al., 2022; Vassilieva & Nguyen, 2023).

Captured Anurans in Natural Pond (NP) and Artificial Pond (AP)

From Table 3, NP II had the highest number of captured anurans (19 individuals), followed by NP I (18). In contrast, NP V and AP II had no captures. NP III and NP IV each recorded seven individuals, while AP I and AP IV had five, AP V had four, and AP III had two. These variations highlight differences in anuran abundance across ponds, with NP II emerging as the most populated.

NP II, the largest pond surveyed (2000 m²), exhibited features conducive to anuran diversity, such as shallow depth (15–30 cm), proximity to trails (5–10 m), and light penetration, which can influence calling activity and reproductive success (Kobisk & Kwiatkowski, 2023; Touchon & Warkentin, 2008). Despite its size, NP II had the most acidic pH (6.22), supporting species adapted to such conditions. Pond area, light penetration, and water pH significantly influence amphibian detection probability and reproductive success (Feldman et al., 2023).

The absence of anurans in NP V and AP II was likely due to habitat disturbances and structural limitations. NP V, frequented by the local Orang Asli community, experiences human activity that may disrupt anuran populations (Aureo et al., 2019). AP II, located on steep terrain and surrounded by dense canopy cover (416.29 ± 253.02 lux), had limited light penetration and leaf litter accumulation, making it unsuitable for anurans (Sánchez-Ochoa et al., 2020).

Artificial ponds utilize black trays (98 cm × 67 cm × 9 cm), which pose minimal risk to amphibians compared to deeper artificial ponds, where mortality risks are higher due to trapping (Wei et al., 2023). Most artificial ponds monitored in this study pose medium to high risks due to persistent high water levels during rainy seasons.

In natural ponds, species such as *Kalophrynus palmatissimus*, *Leptobrachium nigrops*, *Microhyla berdmorei*, and *M. mantheyi* predominantly occupied the forest floor. In artificial ponds, anurans were concentrated in microhabitats such as forest litter and sandbanks, highlighting their habitat-specific preferences (Table 3).

Table 3
Number of anuran individuals caught at each pond

Pond	No. Individual (n)	No. of Species (%)
Natural pond (NP)		
NP I	18	6 (38%) *Hn, Hl, Iq, Lb, Ol, Hg*
NP II	19	9 (56%) *Hn, Kp, Lm, Ln, Mb, Mm, Ol, Hg, Rp*
NP III	7	3 (19%) *Kc, Kp, Hl*
NP IV	7	5 (31%) *Hn, Hl, Fc, Ln, Np*
NP V	0	0 (0%)
Artificial pond (AP)		
AP I	5	3 (33%) *Fc, Kp, Ol*
AP II	0	0 (0%)
AP III	2	2 (22%) *Kp, Lm*
AP IV	5	4 (44%) *An, Dm, Kp, Hl*
AP V	4	3 (33%) *Lb, Lm, Hg*

Note. *Dm: Duttaphrynus melanostictus, Fc: Fejervarya cancrivora, Hg: Hylarana glandulosa, Hl: Hylarana labialis, Hl: Hylarana laterimaculata, Hn: Hylarana nicobariensis, Iq: Ingerophrynus quadriporcatus, Kc: Kurixalus chaseni, Kp: Kalophrynus palmatissimus, Lb: Limnonectes blythii, Lm: Limnonectes malesianus, Ln: Leptobrachium nigrops, Mb: Microhyla berdmorei, Mm: Microhyla mantheyi, Np: Nyctixalus pictus, Ol: Occidozyga laevis, Pl: Polypedates leucomystax,* and *Rp: Rhacophorus pardalis*

Anuran Family and Their Habitat Preference

The study documented anuran species across various families, revealing distinct habitat preferences that reflect their ecological adaptability. The family Bufonidae included *Duttaphrynus melanostictus* and *Ingerophrynus quadriporcatus*. *D. melanostictus*, observed near disturbed landscapes and lowland regions, showed adaptability to bare soil and grass areas, with breeding occurring in artificial water bodies due to their ability to retain water, consistent with findings by Nadia (2020) and Hawkeswood and Sommung (2017). In contrast, *I. quadriporcatus* was associated with swamp forests and artificial habitats, such as plantations, relying on stagnant water for reproduction, which indicates vulnerability to habitat disturbances (Chan-ard et al., 1999; IUCN, 2024). The family Ranidae, represented by *Hylarana glandulosa*, *H. labialis*, *H. laterimaculata*, and *H. nicobariensis*, exhibited the highest species richness (22 individuals), predominantly near NP I, characterized by enclosed areas with palm trees. These ground-dwelling frogs adapt to a range of habitats but show a preference for forested plots, which provide necessary vegetation and microhabitats, as reported by Blackburn and Wake (2011) and Bahiah et al. (2019). Within the family Megophrynidae, *Leptobrachium nigrops* individuals were found near a natural breeding pond, highlighting their reliance on primary rainforest leaf litter and shallow forest streams for reproduction, with large, black tadpoles as a distinctive feature (Ecology Asia, 2024).

The family Microhylidae included *Kalophrynus palmatissimus*, *Microhyla berdmorei*, and *M. mantheyi*, totalling 15 individuals. *Kalophrynus palmatissimus* demonstrated exceptional camouflage and a preference for undisturbed lowland rainforests, while *Microhyla* species were associated with waterlogged grasslands and leaf litter habitats, emphasizing the importance of these microhabitats for their survival (Faris et al., 2019). The family Dicroglossidae comprised species such as *Fejervarya cancrivora*, *Limnonectes blythii*, and *Occidozyga laevis*, with 18 individuals recorded. These species showed habitat preferences ranging from marshy areas and small puddles to disturbed environments like plantations and forest trails (Jaafar et al., 2012; Klys, 2011). Notably, *O. laevis* thrives in aquatic microhabitats such as small streams and puddles, underscoring the critical role of water availability for breeding activities (Semlitsch & Bodie, 2003). The family Rhacophoridae, represented by *Kurixalus chaseni*, *Nyctixalus pictus*, *Polypedates leucomystax*, and *Rhacophorus pardalis*, included seven individuals. *Kurixalus chaseni* and *N. pictus*, newly identified in AHFR, preferred disturbed and primary forests (Gillespie et al., 2021). *Polypedates leucomystax* exhibited high adaptability to non-forested and restoration areas, often perching on shrubs and trees (Nadia et al., 2022), while *R. pardalis* demonstrated seasonal ground-level activity during reproduction, highlighting its primarily arboreal nature (Shahriza et al., 2011).

The study found higher diversity in natural breeding ponds ($H' = 2.47$, $S = 16$) compared to artificial ponds ($H' = 1.98$, $S = 9$) (Table 4), although evenness was comparable. Natural ponds support a wider range of species, including those less adaptable to artificial habitats. This aligns with Hazell et al. (2003), who emphasized the importance of conserving natural water bodies as they sustain species that rely on specific habitat conditions. These findings underscore the necessity of habitat conservation, particularly for anurans that depend on undisturbed ecosystems, to ensure the long-term survival of these ecologically significant species.

Table 4
Diversity indices of anurans

Parameter	Natural Breeding Pond	Artificial Breeding Pond
Total number of species (S)	16	9
Individuals (n)	51	16
Population mean (μ)	3.19	1.78
Shannon-Weiner index (H')	2.47	1.98
Evenness (E)	0.89	0.90

The diversity indices presented in Table 4 highlight a difference in species richness between natural and artificial ponds. The population mean (μ), representing the average number of individuals per species, is 3.19 in the natural breeding pond and 1.78 in the artificial pond, highlighting the superior ecological conditions of the natural habitat. Natural ponds, with their more complex and stable environments, offer a wider range of microhabitats and resources, supporting a greater diversity of anuran species compared to the simpler and more variable conditions of artificial ponds (Aureo et al., 2019; Feldman et al., 2023). In contrast, the artificial pond's lower population mean suggests reduced ecological suitability due to simplified structures and altered conditions, likely impacting species survival and reproduction. This disparity underscores the negative impacts of habitat modification on amphibian communities (Kabanze et al., 2024; Stuart et al., 2004). Conserving natural breeding habitats is essential for sustaining amphibian diversity and ensuring ecological stability.

Despite this difference in species richness, the evenness values for both types of ponds are high, approaching a value of one. This suggests a relatively uniform distribution of species abundance within each habitat, with no single species dominating the community. This reflects the stable ecological conditions maintained by both natural and artificial ponds, providing a balanced environment where each species can thrive without a significant competitive advantage or disadvantage (Touchon & Warkentin, 2008; Sánchez-Ochoa et al., 2020). Interestingly, the high evenness in the artificial pond may be due to the low species richness (S = 9) and total number of individuals (n = 16), which can lead to an artificial balancing effect where few individuals are distributed evenly among fewer species. Conversely, the natural pond, despite having a slightly lower evenness, supports a significantly higher number of species (S =16) and individuals (n =51), which may result in minor variations in species abundance, reflecting a more dynamic and competitive ecological setting. The data suggest that while the artificial pond appears to lack strong species dominance, this might be attributed to its reduced biodiversity and overall population size rather than ecological balance. In contrast, the natural pond's high species richness and moderate evenness indicate a more complex ecosystem where interspecies interactions and resource availability shape community structure.

Microclimate

This study examined nine microclimatic parameters: surrounding temperature (°C), air humidity (%), soil pH, soil moisture, wind speed (mph), light intensity (lux), water temperature (°C), water pH, and dissolved oxygen (mg/L). Due to nocturnal sampling in a forested area, light and wind consistently registered as zero. Table 5 presents differences in microclimatic conditions between natural and artificial ponds. Natural ponds had a slightly more acidic pH (mean: 6.62) compared to artificial ponds (mean: 7.00). Additionally, natural ponds exhibited higher water temperatures (27.00 °C) but cooler surrounding temperatures (29.70 °C) than artificial ponds (water temperature: 26.75 °C, surrounding temperature: 29.80 °C). Natural ponds also had higher dissolved oxygen and humidity levels (mean: 75.5%) compared to artificial ponds (mean: 73.2%). The natural pond area has lower light intensity (mean: 1512.5 lux) than artificial ponds (mean: 1646 lux). A t-test between the two pond types yielded a p-value below 0.05, indicating significant differences in their microclimates (Table 6).

Natural ponds generally offer more favourable environmental conditions for anuran species, including lower pH, higher water temperatures, cooler air temperatures, higher dissolved oxygen levels, increased humidity, and lower light intensity. These conditions are critical for anuran reproduction, as temperature, humidity, and light influence hormonal processes essential for breeding (Browne & Edwards, 2003). Rising temperatures, for example, can enhance feeding activity and metabolic processes, thus impacting frog abundance and productivity (Browne & Edwards, 2003; Carvalho-Rocha, 2020). Humidity is particularly crucial, as desiccation is a major threat to anuran survival. Species without parental care must select breeding sites with adequate humidity to avoid desiccation and ensure successful development (Angiolani-Larrea et al., 2024).

Soil moisture also plays a key role in amphibian survival, as moist conditions are necessary to maintain skin hydration for efficient gas exchange (Hoffmann et al., 2021). Studies show that higher soil moisture enhances frog survival, especially during the juvenile stage, and that damp conditions are sufficient for frog presence (Aryal et al., 2020; Haggerty et al., 2019). Additionally, correlation analysis revealed a significant relationship between humidity and the abundance of *Hylarana labialis* (p-value = 0.028), while no significant differences were found for *Kalophrynus palmatissimus* across other microclimate factors.

Ceron et al. (2020) highlighted that species composition within the Atlantic Forest's metacommunity fluctuates independently, influenced by seasonal temperature, rainfall, and humidity patterns. Similarly, Cicchino et al. (2020) found that air temperature and humidity impact water loss in frogs and frogs call more frequently when humidity and temperature are higher. These findings align with broader phenological patterns, emphasizing the importance of environmental cues in anuran reproductive success (Canavero et al., 2019; Chmura et al., 2019; Post, 2019).

In correlation analyses, environmental temperature showed significant differences with humidity (p-value = 0.003), dissolved oxygen (p-value = 0.011), water temperature (p-value = 0.005), and water pH (p-value = 0.041). Dissolved oxygen differed significantly from the other factors, except for light. Although *Hylarana labialis* and *Kalophrynus palmatissimus* showed correlations with environmental temperature, humidity, soil pH, and soil moisture, it is important to note that correlation does not imply causation, as other factors may influence the observed relationships (Gogtay & Thatte, 2017).

Table 5
The range and mean of microclimate data of the natural and artificial breeding ponds

Pond	Water pH	Water Temp. (°C)	Env. Temp. (°C)	DO (mg/L)	Soil pH	Light (lux)	Humidity (%)	Wind (mph)
	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
NP I	6.62 ± 0.60	25.58 ± 0.71	29.35 ± 1.77	4.46 ± 1.93	6.00 ± 0.67	499.33 ± 234.39	78.62 ± 4.39	0.00 ± 0.00
NP II	6.22 ± 0.56	25.59 ± 0.72	29.48 ± 1.78	5.59 ± 2.24	6.51 ± 0.38	383.72 ± 188.45	78.47 ± 4.15	0.00 ± 0.00
NP III	6.42 ± 0.84	25.93 ± 0.98	29.51 ± 1.66	6.30 ± 4.39	6.10 ± 0.76	799.65 ± 636.80	77.33 ± 4.95	0.00 ± 0.00
NP IV	6.62 ± 0.65	26.01 ± 0.88	29.51 ± 1.59	6.72 ± 3.50	6.40 ± 0.36	383.47 ± 193.63	79.30 ± 3.81	0.00 ± 0.00
NP V	6.70 ± 0.68	26.04 ± 0.76	30.11 ± 1.72	6.20 ± 2.35	6.32 ± 0.21	984.73 ± 2.38	78.27 ± 4.17	0.00 ± 0.00
NP Mean	6.52 ± 0.19	25.83 ± 0.23	29.59 ± 0.30	5.86 ± 0.88	6.26 ± 0.21	610.18 ± 269.79	78.40 ± 0.71	0.00 ± 0.00
AP I	7.01 ± 0.67	26.48 ± 1.30	29.86 ± 1.62	6.49 ± 2.78	0.00 ± 0.00	1572.89 ± 893.65	79.10 ± 3.55	0.00 ± 0.00
AP II	7.08 ± 0.78	25.51 ± 0.80	29.89 ± 1.71	6.72 ± 3.50	0.00 ± 0.00	416.29 ± 253.02	75.90 ± 6.50	0.00 ± 0.00
AP III	7.14 ± 0.73	25.74 ± 0.87	29.94 ± 1.75	6.10 ± 2.41	0.00 ± 0.00	680.82 ± 468.56	76.49 ± 5.76	0.00 ± 0.00
AP IV	6.94 ± 0.65	25.89 ± 0.84	30.04 ± 1.55	6.49 ± 3.40	0.00 ± 0.00	541.47 ± 272.95	76.48 ± 5.27	0.00 ± 0.00
AP V	6.90 ± 0.67	26.05 ± 1.20	29.62 ± 1.71	6.92 ± 3.35	0.00 ± 0.00	177.12 ± 123.94	78.96 ± 3.80	0.00 ± 0.00
AP Mean	7.01 ± 0.10	25.94 ± 0.37	29.87 ± 0.15	6.54 ± 0.31	0.00 ± 0.00	677.72 ± 533.58	77.39 ± 1.52	0.00 ± 0.00

Note. Env: Environment, temp: Temperature, NP: Natural Pond, AP: Artificial Pond

Table 6
Comparative analysis of microclimate variables between natural and artificial breeding ponds with t-test results

	Mean Natural Pond	Mean Artificial Pond	t-value	df	*p*-value
Water pH	6.62 ± 2.38	7.00 ± 0.69	19.11	9	0.000
Water Temperature (°C)	27.00 ± 9.11	26.75 ± 1.06	55.88	9	0.000
Environment Temperature (°C)	29.70 ± 3.16	29.80 ± 1.65	78.67	9	0.000
Dissolved Oxygen (mg/L)	9.97 ± 3.41	8.16 ± 3.06	4.13	9	0.003
Light (lux)	1512.50 ± 462.10	1646.00 ± 650.12	2.69	9	0.025
Humidity (%)	75.50 ± 4.30	73.20 ± 5.19	31.36	9	0.000

Microhabitat

The study identified four primary microhabitats utilized by anurans: bare ground (49.51%), forest litter (28.16%), tree surfaces (11.65%), and pond or swamp areas (10.68%). Bare ground was the most frequently observed microhabitat, likely due to the terrestrial nature of most species recorded, belonging to families such as Bufonidae, Dicroglossidae, Microhylidae, and Megophrynidae. Arboreal species, such as those in Ranidae and Rhacophoridae, were less frequently captured, possibly due to their inherent elusiveness (Bahiah et al., 2019).

Forest litter was the second most utilized microhabitat, primarily associated with species from Megophrynidae and Microhylidae. Megophrynidae species, such as litter frogs, are well adapted to the forest floor, where their camouflage enhances survival (AmphibiaWeb, 2024). Similarly, the fossorial and terrestrial tendencies of Microhylidae, particularly *Kalophrynus palmatissimus* (12 individuals recorded), align with previous findings, highlighting their preference for litter-rich habitats (Badli-Sham et al., 2023; Nadia, 2017).

Forest litter provides critical ecological benefits, such as increased soil moisture conservation, shade, and stable humidity (Jourgholami et al., 2022), which are essential for species such as *Microhyla* sp. For example, *Microhyla annectens* species thrive in environments with 75%–95% litter coverage, conditions that help maintain hydration and activity levels under optimal temperature and humidity (Nadia et al., 2022).

Tree surfaces and pond/swamp areas were less frequently utilized, suggesting that they were either less suitable or less accessible for the majority of the recorded species. These findings reinforce the importance of maintaining diverse microhabitats to support anuran populations, particularly in light of habitat modification that could alter microhabitat availability and quality (Bahiah et al., 2019). Conservation strategies should prioritize the protection of heterogeneous microhabitats to preserve anuran biodiversity and ecological functionality.

Tadpole

Tadpole species observed in the ponds included *Hylarana labialis*, *Kalophrynus palmatissimus*, and *Microhyla mantheyi*. Tadpoles of *H. labialis* were recorded in natural ponds NP I and NP IV, while *K. palmatissimus* and *M. mantheyi* were observed in artificial pond AP I. Complete metamorphosis was documented for *H. labialis* and *K. palmatissimus* in their respective ponds. Interviews with forest officers revealed that *K. palmatissimus* primarily lays eggs in natural temporary ponds characterized by stagnant water and abundant leaf litter (Mohd Naeem Abdul Hafiz, personal communication, July 17, 2022). Interestingly, this species also utilized the artificial ponds during its larval stage.

Tadpoles were observed in two natural ponds (NP I and NP IV) and one artificial pond (AP I). Natural ponds, with sizes ranging from 120–2000 m², provided a more intricate habitat structure compared to the smaller artificial pond (0.66 m²), which likely contributed to higher habitat diversity and more resources for the tadpoles. The larger area of natural ponds allowed for varying depths, substrates, and vegetation, which catered to the specific needs of different species (Hiragond & Saidapur, 2001). Additionally, the presence of leaf litter in natural ponds increases substrate complexity, moisture retention, and nutrient cycling, all of which are essential for tadpole development (Song et al., 2021). In line with these findings, Camacho-Rozo and Urbina-Cardona (2021) demonstrated that natural ponds support higher species richness, greater larval abundance, and more significant spatial and temporal turnover compared to anthropogenic water bodies, emphasizing the ecological advantages of natural habitats.

The natural ponds hosted protists, algae, and *Closterium* sp., forming a balanced food web for tadpoles. In contrast, artificial ponds contained mosquito larvae, dragonfly nymphs, and algae but had fewer microorganisms, thereby limiting nutritional resources. The presence of dragonfly nymphs, known predators of tadpoles, further heightened predation risks in artificial ponds (Kruger & Morin, 2020). Studies on tadpole diets indicate algae and protozoa are dominant food sources (Santos et al., 2016), suggesting that natural ponds may better support tadpole growth due to richer microbial diversity.

The artificial pond (AP I) hosted tadpoles of *Kalophrynus palmatissimus* and *Microhyla mantheyi*, indicating their adaptability to artificial habitats. However, natural ponds supported a single species, *Hylarana labialis*, likely due to their more stable environmental conditions and larger area, which reduces interspecific competition and predation risks. Tadpoles of *H. labialis* are generally larger compared to *K. palmatissimus* and *M. mantheyi*, possibly requiring a larger pond to support their developmental and survival needs. Furthermore, the constant water availability during the seasonal rainfall patterns, as observed in September, may synchronize anuran reproductive behaviours with pond availability and environmental stability, highlighting the critical role of climatic cues in amphibian life cycles (Canavero et al., 2019; Llusia et al., 2013;).

Leaf litter decomposition, driven by microbial activity and shredders, is a crucial ecological process for nutrient cycling and detritus formation in natural ponds (Iwai et al., 2009; Montaña et al., 2019). Tadpoles, as primary consumers, contribute significantly to the breakdown of organic matter, supporting nutrient cycling and aquatic food webs (Montaña et al., 2019). In contrast, the newly established artificial ponds lack the accumulated organic matter found in older natural ponds, which have had years of decomposition (Iwai et al., 2009). Additionally, the absence of key shredders, such as certain invertebrates and tadpole species, further hinders decomposition in these artificial environments.

Future studies should extend sampling periods to capture diverse breeding seasons and evaluate how habitat complexity, pond size, and predator-prey interactions influence the survival and development of tadpoles. Understanding these dynamics will aid in the conservation of anuran populations and their critical roles in freshwater ecosystems.

CONCLUSION

The natural breeding ponds demonstrate higher species diversity compared to artificial breeding ponds. *Kalophrynus palmatissimus*, an endemic and near-threatened species, exhibited the highest species abundance across both natural and artificial breeding ponds. The successful utilization of our artificial pond shows its potential to become an alternative breeding site for anurans in forested areas.

This research has been successful in benefiting the conservation of anuran species, particularly those classified as endangered or near threatened, across both breeding ponds. For future research, ensuring adequate depth of artificial ponds to prevent water overflow during heavy rains is critical. Expanding the sampling area and duration to encompass a broader range of anuran habitats and reproduction periods would also be advantageous. To enhance anuran populations, establishing additional artificial ponds is recommended to increase the availability of suitable oviposition sites. Increasing the number of these breeding habitats can promote greater anuran abundance and contribute to population resilience. This approach may mitigate the effects of habitat loss and fragmentation, supporting the long-term conservation of amphibian communities.

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The Thermogenic Effect of Reproductive Hormones in Female Bornean Orangutans (*Pongo pygmaeus*) at Bukit Merah Orang Utan Island, Perak

Noramira Nozmi[1], Farida Zuraina Mohd Yusof[2]*, Hartini Ithnin[3], Muhammad Fahmi Ramli[4] and Nur Nadiah Md Yusof[1]

[1]*Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), 40450 Shah Alam, Selangor, Malaysia*
[2]*Integrative Pharmacogenomics Institute (iPROMISE), Universiti Teknologi MARA (UiTM) Selangor Branch, 42300 Bandar Puncak Alam, Selangor, Malaysia*
[3]*Department of Wildlife and National Parks, KM 10, Jalan Cheras, 56100 Kuala Lumpur, Malaysia*
[4]*Bukit Merah Orang Utan Island Perak Foundation, Bukit Merah, 34400 Simpang Empat Semanggol, Perak, Malaysia*

ABSTRACT

Basal body temperature (BBT) is commonly linked to progesterone and oestrogen concentration, two key reproductive hormones in female humans and non-human primates. Retrospectively, little attention has been given to investigating the association between basal body temperature and reproductive hormones via non-invasive methods among orangutans. Hence, this study was carried out to identify the associations between basal body temperature and reproductive hormones via a non-invasive approach among four female orangutans at Bukit Merah. The basal body temperature of two adult females, Baboon (age 33) and Careena (age 15) and two adolescent female orangutans, April (age 13) and Kate (age 9), was measured using an infrared thermometer gun. A total of 101 faecal samples were gathered and analysed via enzyme-linked immunosorbent assay (EIA). A Spearman's correlation coefficient test revealed a significant moderate positive relationship between progesterone and basal body temperature with r[101]=0.437, p=0.041. Nevertheless, there was no significant relationship between basal body temperature and oestradiol. These findings align with previous research conducted on other mammals, which has shown that progesterone elevation is associated with increased body heat. This result also demonstrated the reliability of using non-invasive techniques, such as infrared thermography and faecal hormone analysis, for tracking reproductive hormone changes in orangutans. This study contributes valuable data to understand the physiological effects of progesterone on thermoregulation in female

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E-mail addresses:
miranozmi@outlook.com (Noramira Nozmi)
fzuraina@uitm.edu.my (Farida Zuraina Mohd Yusof)
hartini@wildlife.gov.my (Hartini Ithnin)
fatehmie@gmail.com (Muhammad Fahmi Ramli)
nurnadiah@uitm.edu.my (Nur Nadiah Md Yusof)
*Corresponding author

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orangutans and offers practical insights for captive breeding programs aimed at enhancing orangutan welfare. Future research could explore the broader implications of progesterone's thermogenic effects on behaviour and reproductive success.

Keywords: Luteal phase, reproductive hormone, resting body temperature, sex steroid hormone

INTRODUCTION

Bornean orangutans (*Pongo pygmaues*) have been listed by the International Union for Conservation of Nature and Natural Resources (IUCN) Red List as one of the critically endangered species (Ancrenaz et al., 2016). They are recognized as the world's largest arboreal mammals and are known for their distinctive behaviour, physiology and reproductive biology (Ancrenaz et al., 2014). Just like humans, female orangutans have complex reproductive cycles that are controlled by multiple hormone fluctuations (Knott, 1999). One of the main hormones is progesterone, which plays a significant role in preparing the body for conception and maintaining pregnancy (Halasz & Szekeres-Bartho, 2013). Estrogen is another key hormone that is responsible for developing female sexual characteristics (Delgado & Lopez-Ojeda, 2023). Despite extensive research on the reproductive physiology of orangutans, limited studies have been conducted on how hormonal changes can affect body temperature regulation in orangutans. Bukit Merah Orang Utan Island serves as an important conservation and research centre for Bornean orangutans, providing a controlled environment to study these physiological processes in detail.

The link between basal body temperature and reproductive hormones has been well-studied in humans since the 1940s (Écochard et al., 2022; Rose & Jones, 1996). Nevertheless, there is still a significant interest in determining the relationship among wildlife, particularly among orangutans. In humans, elevated basal body temperature, in tandem with progesterone concentration, is used to track ovulation or pregnancy (Écochard et al., 2022). This subtle temperature shift is a valuable indicator in assessing hormonal changes and fertility (Écochard et al., 2022). While this association has been well-studied in humans, its role in other primates, particularly orangutans, remains largely unexplored.

The only research that had been done among orangutans was by Asa et al. (1994) 30 years ago. Though the study established a significant relationship between progesterone and basal body temperature, the method used was ethically questionable (Asa et al., 1994). Asa et al. (1994) measured basal body temperature peritoneally by inserting radio telemetry transmitters. The insertion procedure required restraining the animals and sedation, which caused stress for the orangutans (Asa et al., 1994). Stress significantly impacts hormone concentration, thus causing inaccurate results reflected in the biological samples (Foley et al., 2001).

Hence, this study aims to investigate the thermogenic effects of reproductive hormones in orangutans using commercial infrared thermometer guns as a non-invasive approach to understand their physiology and its implications for future breeding programs. Basal body temperature recording using a thermometer gun is the simplest and easiest approach, without disturbing the animals or causing stress to them (Su et al., 2017). In addition, it is also a safer approach for the researcher as temperature recording requires a distance from the animal. Additionally, this research will contribute to the expansion of knowledge on the relationship between hormones and basal body temperature in primates, offering valuable insights into how these mechanisms have evolved across species.

METHODS

Sampling Site

This study was conducted at Bukit Merah Orang Utan Island, an ex-situ conservation centre that housed 20 orangutans in a 14-acre land area. Bukit Merah is situated approximately 284 kilometres from the centre of Kuala Lumpur, requiring a four-hour drive from the city. The island is surrounded by abundant tropical lowland rainforest trees such as the Bertam palm tree (*Eugeissona tristis*), *asam gelugur* (*Garcinia atroviridis*) and fruit trees (Dharmalingam et al., 2012). *Rambutan* (*Nephelium hamulatum*) and *pulasan* (*Nephelium rambutan-ake*) are examples of fruit trees found on the island (Dharmalingam et al., 2012). The climate and flora of the island are notably comparable to the ecological niche of orangutans in Borneo and Sumatra, Indonesia (Dharmalingam et al., 2012).

Animals, Facilities and Management

The island housed 20 Bornean orangutans in a 19.6 m^3 individual enclosure area during the night. In the morning, orangutans are released into the exhibit areas. Four female orangutans were involved: Baboon (age 33), Careena (age 15), April (age 13) and Kate (age 9) (Figure 1). The selected subjects were divided into two age categories: adults (Baboon and Careena) and adolescents (April and Kate). Orangutans of Bukit Merah are fed with seasonal fresh fruits, approximately 500 g per individual daily during the day and another 500 g during the evening. Additionally, vitamin drinks and fruit juices were also given as daily supplements, with ad libitum water available. Enclosure areas are cleaned and sanitized daily after the animals are released to their respective exhibit areas.

Data Collection

This study was conducted from March 2021 to June 2021, with daily recording of basal body temperature and faecal collection upon availability.

Basal Body Temperature Recording

A Fisherbrand™ Traceable™ Infrared Thermometer Gun was used to record basal body temperature. The thermometer gun can detect temperature within -50 °C to +1000 °C with

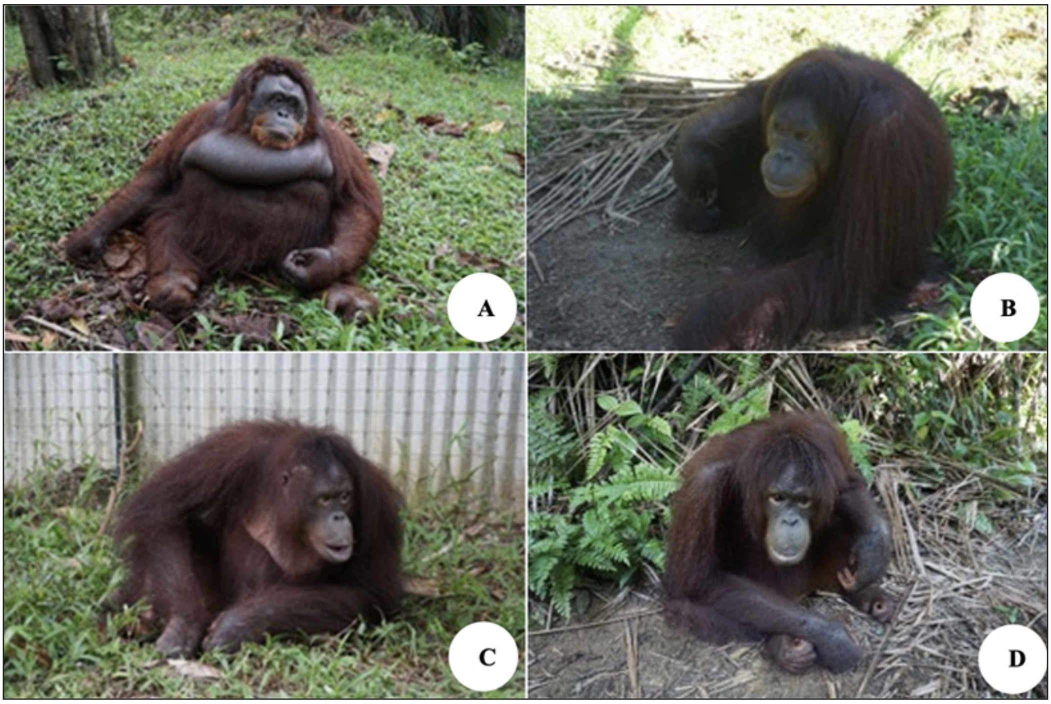

Figure 1. Female orangutans at Bukit Merah Orang Utan Island, Perak. A. Baboon (age 33). B. Careena (age 15). C. April (age 13). Kate (age 9)

resolution at 0.1 °C to 200 °C, 1 °C over 200 °Cand accuracy range at ±1.5% + 2 °C. Basal body temperature was recorded at a similar time in the morning to ensure the accuracy of the results. Three readings were taken and then averaged to get the final temperature. The temperature was recorded by aiming the gun directly at the forehead.

Progesterone Hormone Analysis

Faecal Sampling. Faecal samples were collected based on availability. A total of 101 faecal samples were collected from the orangutans. Enclosures were cleaned daily to minimize faecal contamination and to keep the area hygienic for the animals. Sterile gloves and a mask are mandatory to be worn prior to sample collection to prevent sample contamination. Only fresh samples free from foreign materials were selected and stored in a labelled container. The name of the individual and the date were labelled on the container and placed in a cooling box immediately. The samples were then transferred to a freezer located on the jetty at -20 °C until further analysis. All samples were analysed at Wildlife Forensic Lab, PERHILITAN Headquarters, Cheras, Kuala Lumpur.

Faecal Hormone Extraction

Hormone extraction from the faecal samples started with oven drying at 55 °C. Pestle and mortar were used to powder the dried samples afterward. Next, 0.2 g of the powdered faecal samples was mixed with 2 mL of ACS-graded ethanol in a tube. The tube was then shaken for 30 minutes at room temperature and centrifuged at 5000 rpm for 15 minutes at 4 °C using a Thermo Scientific Heraeus Multifuge X1R centrifuge. Lastly, the supernatants were transferred into a microtube and stored in a freezer at -20 °C. The extraction analysis then proceeded into EIA analysis according to the procedures by Arbor Assays DetectX® Progesterone and Estradiol Enzyme Immunoassay.

Statistical Analysis

This study used IBM SPSS version 23.0 to analyse the data, and significant differences were noted when the p-value was <0.05. Descriptive analysis was used to determine median and interquartile range (IQR) values. Due to the small sample size (n<10), data were analysed non-parametrically. Spearman's correlation test was applied to test the relationship between sex steroid hormones and the basal body temperature of orangutans. The correlation strength follows the guideline from Ratner (2009), which indicates a weak linear relationship for the range between 0 and ±0.3, a moderate linear relationship for the range ±0.3 to ±0.7, and a strong relationship for the range between ±0.7 and 1.0.

RESULTS

Basal Body Temperature-Reproductive Hormone Relationship

Table 1 shows a significant moderate positive relationship between progesterone and basal body temperature with r[101] =0.437, p=0.041 (Table 1). Interestingly, there is no significant relationship between oestradiol and basal body temperature, with a p-value >0.05 (Table 1).

Table 1
The correlation between basal body temperature and progesterone concentration of female orangutans at BMOUI, Perak, using Spearman's correlation coefficient test

	Basal Body Temperature		**Strength of correlation**
	r	**p-value**	
Progesterone	0.437	0.041*	Significant moderate positive linear relationship
Estradiol	-0.077	0.445	Non-significant weak negative linear relationship

Note. **P-value is highly significant at p<0.001, *P-value is significant at p<0.05

DISCUSSION

In this study, a higher concentration of progesterone is significantly linked to basal body temperature and vice versa (Table 1). This finding is consistent with the studies among female orangutans and humans, which found that pregnanediol-3 alpha-glucuronide (PdG) is also positively related to BBT (Asa et al., 1994; Écochard et al., 2022). Additionally, previous research has also confirmed that the increase of progesterone elevates the basal body temperature, confirming ovulation in females (Su et al., 2017).

On the contrary, chimpanzees showed no significant relationship between basal body temperature and progesterone, but it is associated with oestrogen in tandem with the swelling of genital areas (Graham et al., 1977). The swelling of genital areas and a fall in temperature a day after oestrogen peaks, marking the beginning of a fertile phase in chimpanzees (Graham et al., 1977). The swelling of genital areas is non-visible in orangutans and humans, which is a phenomenon called "concealed ovulation" (Durgavich, 2013). This exclusive phenomenon found in only orangutans and humans is a condition where physical cues are absent in determining their fertile phase (Rooker & Gavrilets, 2020). Similarly, Tasmanian bettongs also show a BBT peak during estrous days, followed by a temperature dip in the next two days (Rose & Jones, 1996). On day three, the temperature increased steadily until day 10, together with the rise of progesterone concentration (Rose & Jones, 1996). The temperature dropped two days before oestrus in relation to the decrease of progesterone level and leucocytes observed in vaginal smears (Rose & Jones, 1996). This study demonstrated the adaptability of the BBT method in determining reproductive status across the Mammalia clade, not just in humans and non-human primates.

Basal body temperature is also found to be associated with progesterone among marine mammals such as beluga whales (Katsumata et al., 2006a) and killer whales (Katsumata et al., 2006b). Interestingly, in the studies, the basal body temperature-progesterone relationship was observed among pregnant females and monitored throughout the pregnancy (Katsumata et al., 2006b). It was found that their temperature dropped significantly by 0.3 °C below the mean value five days before parturition and decreased by 0.8 °C a day before parturition (Katsumata et al., 2006b). This finding is valuable in estimating the parturition day for infant delivery preparation for the caregiver or researchers.

Remarkably, the study found that beluga whales demonstrated a similar relationship between basal body temperature and progesterone level, which mimics relationships observed in female orangutans and humans (Katsumata et al., 2006a). In the study, the body temperature of the beluga was low when progesterone concentration declined and increased 11 days after mating took place (Katsumata et al., 2006a). This is evidence that progesterone secreted from corpora lutea is crucial in raising the BBT (Ogino, 1930). It is also worth noting that mating behaviour was observed in beluga whales during low body temperatures (Katsumata et al., 2006a). In summary, this study concluded that the shift of body temperature is linked to the follicular and luteal phases of the estrous cycle in this species. Based on these studies of whales, it is evident that the BBT approach is a flexible and reliable method for determining the reproductive status of marine mammals.

Additionally, similar findings are also found among women taking oral contraceptive pills that contain artificial progesterone (Baker et al., 2001). Body temperature is significantly elevated continuously for 24 hours, suggesting a prolonged effect of synthetic hormone steroids, which is uncommon in the natural menstrual cycle (Baker et al., 2001). This indicates that progestins, a synthetic hormone that is usually combined with oestrogen, have a higher relative binding affinity for the progesterone receptor compared to natural progesterone (Juchem & Pollow, 1990). Additionally, it is also due to the longer metabolic clearance rate compared to natural progesterone (Bergink et al., 1990). Even when the amount of progestins subsides after hormone withdrawal, it could still be active in the brain (Feder & Marrone, 1977) or metabolized into neuroactive steroids (Rogers & Baker, 1997). Due to this effect, women using oral contraceptives had a prolonged thermogenic effect directly or indirectly through progesterone metabolites up to three days after discontinuation (Baker et al., 2001). In menopausal women, progesterone is reported to cause hyperthermia for several days after treatment withdrawal (Piette et al., 1994).

Despite the simplicity and ease of collecting basal body temperature data, interpreting the results can be challenging (Su et al., 2017). This is aligned with the findings from Guermandi et al. (2001) and Hilgers and Bailey (1980), who found that the basal body temperature dip range was too big, from four to eight days prior to ovulation, suggesting that BBT is a poor approach in predicting ovulation in females (Guermandi et al., 2001; Hilgers & Bailey, 1980). In addition, ovulation was determined in only 43% of fertile women and 25% of infertile women, which is insufficient for BBT to be implemented as an ovulation predictor (Lenton et al., 1977). On top of that, scientists have rejected the application of BBT in determining ovulation due to wide day-to-day environmental temperature fluctuation and the influence of illness, medication, diet and changes in sleep patterns in females (McCarthy & Rockette, 1986).

In addition, basal body temperature is also reported to be influenced by environmental parameters, including environmental temperature, rainfall, and duration of daylight (Tatsumi et al., 2020). BBT was also found to be affected by biological factors, including stress, irregular sleep cycles, health issues and drugs (Su et al., 2017). Moreover, the increasing occurrence of aggressive behaviour in female orangutans is also observed in tandem with elevated progesterone levels (Nozmi et al., 2025), possibly due to increases in basal body temperature and air temperature. Aggressive behaviour is a response to uncomfortable conditions, potentially leading to higher stress levels and increasing the risk of injury to both the individual and other animals (Nozmi et al., 2025). In addition, female orangutans exhibited a longer resting duration when the air is hotter and humid, which is another adaptation strategy to negative environmental changes, resulting in lower time spent on activities such as reproduction, feeding, and playing (Nozmi et al., 2023). Hence, these significant negative impact of the external and internal factors on basal body temperature towards the behaviour of orangutans demonstrates the unreliability of BBT in determining the fertile phase in females.

CONCLUSION

This study implies that the elevation of basal body temperature of orangutans is associated with the rise of progesterone concentration. The safety, ease, and simplicity of using a thermometer gun are useful in monitoring the ovarian cycle, especially during the luteal phase when progesterone levels peak. This information provides information on hormone concentrations linked to the basal body temperature of female orangutans since it is difficult to obtain daily basal body temperature from wild orangutans. Deeper knowledge of orangutans' reproductive physiology and ecology is crucial for effective population management in zoos and to support their survival in the wild. Hence, it would be greatly beneficial to determine other factors that may contribute to the concentration of progesterone from different populations of orangutans, such as in other zoos and conservation centres.

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Organic Fertilizer Formulation to Improve the Quality and Yield of *Eleutherine palmifolia* L. (Merr) as an Alley Cropping in Coffee Plantation

Rina Ekawati[1]* and Kikis Rohzizat Zali[2]

[1]*Program Studi Budidaya Tanaman Perkebunan, Politeknik Lembaga Pendidikan Perkebunan, 55222 Yogyakarta, Indonesia*
[2]*Program Studi Pengelolaan Perkebunan, Politeknik Lembaga Pendidikan Perkebunan, 55222 Yogyakarta, Indonesia*

ABSTRACT

Eleutherine palmifolia L. (Merr), or what is known as the Bawang Dayak in Indonesia, is a horticultural group of plants that can be used as a medicinal plant. This plant has many benefits because it contains secondary metabolite compounds, such as flavonoids, phenols, naphthoquinones, and antibacterial and anticancer derivatives. This research aims to explain the response of *E. palmifolia* plants to a combination of organic fertilizers on growth, yield, and total flavonoid content as an alley cropping under coffee trees. This research was implemented with randomized complete block design. Providing organic fertilizer uses a minus-one test, which consists of five treatments, namely: Without organic fertilizer; Complete fertilization (cow manure + guano + rice husk ash); cow manure + guano (without rice husk ash); guano + rice husk ash (without cow manure); and cow manure + rice husk ash (without guano). Each treatment was repeated five times. The application of complete organic fertilizer (cow manure + guano + rice husk ash) produces a higher number of leaves, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, tuber weight per plant (21.5 g), and tuber weight per plot (89.8 g) compared to without fertilization. The application of organic fertilizer produces the flavonoid content of *E. palmifolia* bulbs 1.2 – 1.3 times higher than no fertilization. The average percentage of shade obtained was 90.41% with an average air temperature of around 29.7°C. *E. palmifolia* can be planted under the coffee tree as an alley cropping. Based on the characteristics of the growth and yield of *E. palmifolia*, further research on the farming feasibility analysis needs to be considered.

Keywords: Bioactive content, *E. Palmifolia*, macronutrients, organic, physiology

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E-mail addresses:
rne@polteklpp.ac.id (Rina Ekawati)
kikisrohzizatzali@gmail.com (Kikis Rohzizat Zali)
*Corresponding author

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INTRODUCTION

The body needs medicinal plants to maintain or improve its immune system, so it is not easily attacked by disease (Adelayanti, 2020). The body's immune system fights viruses and diseases and produces antibodies (Chowdury et al., 2020). One type of medicinal plant widely cultivated or cultivated, especially by the people of Central Kalimantan, is *Eleutherine palmifolia*. This plant is native to tropical America and has many health benefits (Mutiah et al., 2019). *E. palmifolia* also has other regional names, including "bawang sabrang" (Sunda), "bawang Tiwai" (Kutai Kartanegara), "bawang hutan" (East Kalimantan), "brambang sabrang" (Java), and "si marbawang-bawang" (North Sumatra) (Poerwosusanta et al., 2019). *Eleutherine palmifolia* is a medicinal plant because it contains several secondary metabolite compounds, including flavonoids as antioxidants that can be used to improve the immune system (Chabib et al., 2018); flavonoids and phenols as antibacterials (Fransira et al., 2019, 2020); and antiacne (Syamsul et al., 2015), antimitotic (Efendi et al., 2015), antifungal (Christoper et al., 2017), and also antioxidants (Andryani et al., 2022; Kuntorini et al., 2016). Several phytochemicals in medicinal plants include flavonoids, naphthoquinones, anthraquinones, alkaloids, saponins, tannins, triterpenoids, and steroids (Kamarudin et al., 2021; Gomes et al., 2021; Yanti et al., 2022). The central part of this plant is the tuber, which is often used as an herbal medicine and consumed either fresh or dried (simplistic).

Fertilization is one way to increase the availability of nutrients in the soil. Nutrients are needed for plant growth and development. The use of organic fertilizer in this research uses common fertilizers, namely cow manure, guano fertilizer, and rice husk ash, each of which is a source of the macro and micronutrients, such as: Nitrogen (N), Phosphorus (P), and Potassium (K), Fe, Cu, Zn, Mn, Mg, and Ca. The macro and micronutrients can promotes of mineralization (Emendu et al., 2021). The N element stimulates the growth of roots, stems, and leaves. Nutrient N is central in cell division, tiller formation, and stem elongation (Saleem et al., 2012). Plants need N because it plays a role in forming chlorophyll and the growth of stems, branches, and leaves, so it has a significant role in photosynthesis (Leghari et al., 2016). The nutrient P is a component of enzymes and proteins, ATP, RNA, and DNA, and has an essential function in photosynthesis and energy transfer. Chotimah et al. (2021) stated that the application of organic fertilizers can improve the performance and yield of *Eleutherine americana* Merr when grown on acidic tropical soils. Potassium plays a role in root development, increases the formation of sugar and starch in photosynthesis, and is essential in plant translocation. Providing potassium affects the growth and yield of garlic plants. The application of 200 kg K ha^{-1} produces the highest weight of fresh and dry garlic bulbs (Jiku et al., 2020).

Coffee plantation land has the potential for intercrop cultivation. Intercrops are cultivated between rows of annual plants, in this case, coffee plants. Coffee plants usually have a spacing of around 2 m × 2 m (Arabica type) or 2.5 m × 2.5 m (Robusta type). This planting distance allows it to be used for other farming businesses, namely cultivating *E. palmifolia* onion. Optimizing land use in coffee plantation areas can increase farmers' income and soil fertility and create a suitable microclimate for the growth of annual crops. Intercropping has improved coffee production by providing an alternative income source to producers, especially in the crop formation phase (Carvalho et al., 2024).

Cultivation of *E. palmifolia* plants in Indonesia still needs to be developed (Ariska et al., 2020). This research has differences, especially in the methods and land conditions used. The combination of organic fertilization using minus one test treatment aims to explain the response of *E. palmifolia* plants as intercrops in coffee plantations to N, P, and K fertilization because these three nutrients are one of the limiting factors in the growth, yield, and physiology of *E. palmifolia* plants. The minus one test also determines the role of the three nutrients by eliminating one of the nutrients used. Apart from that, the results of this research will also be used to prepare Standard Operational Procedures (SOP) for cultivating *E. palmifolia*. The development of SOPs will require extensive additional or advanced research to generate valid and reliable data

The theoretical benefit of this research is that it can apply technical activities for cultivating seasonal crops on limited land to support an integrated agricultural system. The practical benefit that will be obtained later is using land under the shade of coffee by cultivating annual crops using minimum tillage. This research's managerial benefit is obtaining an organic fertilizer formulation to produce growth, yield, and secondary metabolite levels of *E. palmifolia* plants based on field and laboratory tests, which can later be used as additional information to guide good plant cultivation.

MATERIALS AND METHODS

Study Area and Materials

The research was conducted from December 2022 to July 2023 (8 months) on a 13-year-old coffee plantation. The coffee plantation belongs to the Tunggak Semi Farmers Group in the Pentingsari hamlet, Umbulharjo Village, Cangkringan District, Sleman Regency, Yogyakarta. This location is 662 m above sea level with rainfall of 660 mm/year (Statistics Sleman Regency, 2021).

The materials used in the research included Kalimantan accession *E. palmifolia* bulbs, cow manure, guano phosphate fertilizer, and rice husk ash. The equipment used for cultivation and harvesting are soil processing equipment, oven, and analytical balances. The equipment used to analyse leaf chlorophyll and total flavonoids includes a visible Spectrophotometer (Genesis 30), 100 mL Erlenmeyer flask, measuring flask, filter paper, test tube, and Ohauss analytical balance. Measurement of light intensity under coffee plants uses a lux meter, while air temperature and humidity measurements use a thermohygrometer.

Experimental Design

This research was implemented with randomized complete block design. The treatment given is organic fertilizer. Providing organic fertilizer used a minus-one test which consisted of five (5) treatments, namely: Without organic fertilizer; Complete fertilization (cow manure + guano + rice husk ash); cow manure + guano (without rice husk ash); guano + rice husk ash (without cow manure); and cow manure + rice husk ash (without guano). Each treatment was repeated five times so that there were 25 experimental units.

Procedures

Dayak onion bulbs will first be selected by selecting healthy seeds (shiny colour, compact/ not porous, the skin is not injured, and uniform in size). *E. palmifolia* tubers will be planted between or between the coffee planting distances. The coffee trees on the land have a row spacing of ± 1.9 m, while the row spacing is ± 2.4 m, so the plant population per hectare is ± 2157 plants. The distance between coffee plants is ± 25.5 m. Before planting, the soil under the coffee shade is loosened and mulched first to improve soil aeration and make it easier to plant the tubers. For planted seeds, cut the tip of the tuber by approximately 0.5 cm to help break the dormancy period and accelerate plant growth. Then, the tubers are planted by immersing ¾ of the tubers between or on the sidelines of the coffee plantings.

Applications of cow manure, guano fertilizer, and rice husk ash are given according to the treatment. Guano fertilization will be done 3 days before planting (*pre-plant*) to stimulate the growth of *E. palmifolia*'s roots. Maulidan and Putra (2024) stated that phosphorus can stimulate root growth, especially of lateral and hair roots. Meanwhile, cow manure and rice husk ash will be given two weeks after planting/WAP. The dose of cow manure and rice husk ash used in this study refers to the dose used in research (Yustina et al., 2019) on *E. americana* (Merr.), namely 360 kg ha^{-1}. The dose of guano fertilizer refers to the dose of biophosphate fertilizer used in research (Sukmasari et al., 2020) on shallot plants, namely 200 kg ha^{-1}.

Watering is done at least once daily in the morning/evening or depending on weather conditions. Weeding is done manually once a month. Harvesting will be done five months after planting (MAP). Harvesting *E. palmifolia* at the age of five MAP according to (Rosmawaty et al., 2019), has a single effect on the total flavonoid content of *E. palmifolia* which is the best.

Observation

Observations include components of plant growth and yield, including (1) Plant height observed every week from 4 - 12 WAP with intervals of once every two weeks; (2) The number of leaves observed every week from 4 - 12 WAP with intervals of once every two weeks; (3) Analysis of total flavonoid content (Spectrophotometry method) at 12 WAP; (4) Number of bulbs per plant and fresh weight of bulbs per plant measured at harvest; (5) Bulb weight per plot calculated at harvest; (6) Fresh weight of shoots and roots measured at harvest (five plants); (7) Dry weight of shoots and roots measured after plant biomass was dried using an oven at 60°C for three days; and (8) Microclimates observation.

Measurements of air temperature and sunlight intensity were measured during observations, both under the coffee tree stands and outside the coffee stands. The intensity of sunlight is measured using a lux meter and the air temperature is also read directly on the tool. The formula for calculating the shade percentage is as follows:

Percentage (%) shade = 100% × (1 - I/D)

Note:

I = intensity in the shade

D = intensity outside the shade

The following is an example of calculating the percentage of shade in coffee stands at the experimental location during observations at 4 WAP:

% shading = 100% × (1 - I/D)

= 100% × (1 - 1652.2/3743.2)

= 100% × (1 – 0.4414)

= 100% × 0.5586

= 55.86%

Data Analysis

The data analysis was conducted using the analysis of variance (ANOVA) method and statistically significant differences were determined with the DMRT (*Duncan Multiple Range Test*) at the 5% level with the SAS system for Windows 9.0 (English) tools.

RESULTS AND DISCUSSION

Quality Characteristics of Organic Fertilizer

The analysis of cow manure shows that the C-organic content meets the minimum technical requirements for solid organic fertilizer based on Minister of Agriculture Regulation Number 261 of 2019 concerning minimum technical requirements for organic fertilizers, biological fertilizers, and soil amendments (Table 1). High C-organic content (more than quality standards) is related to the nitrogen mineralization rate, availability of organic material, and microbial decomposers. Providing cow manure can increase the availability of soil organic matter and microbial activity, which helps break down organic matter

(Nuro et al., 2016). The N-total content also meets the minimum technical requirements for organic fertilizer based on Minister of Agriculture Regulation Number 28/Permentan/SR.130/5/2009. Nitrogen determines the C/N ratio in organic fertilizer. The C/N ratio is a good indicator of the N mineralization process in the soil (Rapisarda et al., 2022).

Table 1
Results of analysis of several types of organic fertilizer used in research

Fertilizer type	Test Variable	Result	Minimum Technical Requirements for Fertilizer	Note
Cow Manure	C-organic (%)	29.304	Min. 15	Appropriate-
	N-total (%)	1.090	< 6	Appropriate–Appropriate
Guano	P_2O_5 (%)	2.263	< 6	Appropriate
Rice Husk Ash	K_2O (%)	0.912	< 6	Appropriate

Source: Instiper Laboratory, Yogyakarta (2023)

The Phosphorus (P) nutrient content in guano organic fertilizer meets the minimum technical requirements for organic fertilizer according to Ministry of Agriculture Regulation Number 28/Permentan/SR.130/5/2009 concerning organic fertilizers, biological fertilizers, and soil amendments. Guano fertilizer is fertilizer that comes from bat droppings mixed with soil, which is then composted with the help of microbial activity. Guano fertilizer plays a role in increasing plant growth and stimulating root growth (Muryanto & Lidar, 2020). Phosphorus is needed to development of nucleic acid, energy, and sugar.

Rice husk ash contains the nutrient potassium, which, in this study, the test results met the minimum technical requirements for organic fertilizer according to Minister of Agriculture Regulation Number 28/Permentan/SR.130/5/2009 concerning organic fertilizers, biological fertilizers, and soil amendments. Potassium plays a role in photosynthesis, sugar transportation, protein formation, and enzyme activation.

Soil Analysis Before Planting

The results of the first soil analysis at the experimental location in Table 2 show that several criteria for soil chemical properties, such as C-organic, N-total, C/N ratio, total P_2O_5, total K_2O, and pH provide different criteria. The C-organic content is relatively high, indicating that soil organic matter from plant tissue residues decomposed by decomposing microbes is also high. The experimental land used in this research implemented an organic farming system (without applying inorganic fertilizer) to increase the organic C content, total N, and C/N ratio. The organic C and total N content will also determine the C/N ratio in the soil, which is related to the N mineralization process. A lower C/N ratio indicates that the organic material decomposition process will also run better.

Table 2
The before-planting soil analysis results

Test variable	Result	Standard	Criteria
C-organic (%)	4.173	3 – 5	High
N-total (%)	0.238	0.21 – 0.5	Medium
C/N	17.522	16 – 25	High
P_2O_5 (%)	0.289	> 60 (P_2O_5 HCl 25% (mg/100 g)	Very high
K_2O (%)	0.015		
pH	7,1	6.6 – 7.5	Neutral

Source: Instiper Laboratory, Yogyakarta (2023)

Phosphorus and potassium nutrient levels in this research field are classified as very high. This is thought to be related to the pH value of the soil. The pH value shows neutral. Neutral pH is also related to the availability of nutrients in the soil. The nutrient P has immobile properties in the soil and can be bound by Al and Fe in land conditions with a low pH value (acid). Neutral pH conditions cause P nutrients to be available in the soil so that plants can absorb these nutrients. Land conditions where organic materials have previously been applied are also thought to reduce the ability of metals, such as Al and Fe, to bind P. This can cause Al, Fe, and Mn in the soil solution to decrease as the soil pH value increases to become neutral. This is in line with the research results that adding organic material can improve soil structure, add nutrients, and enhance availability (Tustiyani et al., 2024).

After-Planting Soil Analysis Results

The results of the final soil analysis at the experimental location in Table 3 show that applying organic fertilizer with different treatments provides different soil yield assessments. The criteria for assessing soil chemical properties are based on (Eviati & Sulaeman, 2009). C-organic levels show Medium – Very High values. Application of complete organic fertilization (cow manure + guano + rice husk ash) and cow manure + guano fertilization increased the C-organic value from the initial value (before treatment), namely 4.173% to 6.274% and 4.736%. The increase in the value of C-organic levels is in line with the results of research (Adviany & Maulana, 2019). This is because carbon is the main constituent of organic materials. The more organic material given to the soil, the more organic C released into the soil will increase.

The final N-total content value increased from the initial N-total value (0.238%), except for the complete fertilization treatment (0.223%). The increase in N levels is due to the application of organic fertilizers (cow manure, guano, and husk ash), which have complete macronutrients that are thought to increase the uptake of N nutrients. Apart from that, organic fertilizers also play a role in improving the physical properties of the

Table 3
The final soil analysis results

Test Variable	Treatments			
	Cow Manure + Guano + Rice Husk Ash	**Cow Manure + Guano**	**Guano + Rice Husk Ash**	**Cow Manure + Rice Husk Ash**
C-organic (%)	6,274 (Very high)	4,736 (High)	2,943 (Medium)	4,017 (High)
N-total (%)	0,223 (Medium)	0,492 (Medium)	0,386 (Medium)	0,340 (Medium)
C/N	28,1 (Very high)	9,64 (Low)	7,62 (Low)	11,82 (Medium)
P_2O_5 (%)	0,349 (Very high)	0,348 (Very high)	0,392 (Very high)	0,395 (Very high)
K_2O (%)	0,282 (Very high)	0,497 (Very high)	0,225 (Very high)	0,056 (Very high)
pH	6,30 (Slightly acid)	6,21 (Slightly acid)	6,46 (Slightly acid)	6,69 (Neutral)

Source: Instiper Laboratory, Yogyakarta (2023)

soil so that they can increase the root development of plants (Bachtiar et al., 2020). The highest increase in the C/N ratio value was shown from the complete organic fertilizer treatment (cow manure + guano + rice husk ash), which was 28.1 from the initial C/N ratio value (17.5). In contrast, the C/N ratio value in the fertilizer treatment of other organics experienced a decline. The increase in the C/N ratio value is likely due to decomposing organic material from organic fertilizer applied to the soil. The process of decomposing or composting organic materials requires carbon as an energy source for decomposing bacteria, while nitrogen was one of the macronutrients that played an essential role in the formation of amino acid, nucleic acid, chlorophyll, proteins, and various secondary and primary metabolites. This indicated that the inadequacy of these nutrients could lead to suboptimal growth in plants (Kishorekumar et al., 2020).

After applying organic fertilizer, the phosphorus and potassium levels increased from the initial phosphorus and potassium levels, namely 0.289% and 0.015%. An increase in phosphorus and potassium levels occurred in all organic fertilizer treatments given. Apart from the application of organic fertilizer itself, the increase in phosphorus and potassium levels is also thought to be due to the effect of phosphorus accumulation in the soil from organic fertilization that has been carried out previously. The accumulation of phosphorus nutrients can be caused by the influence of soil pH, which decreases from the initial pH value (7.1). A decrease in the pH value can cause the soil to become slightly acidic, so Al and Fe bind the immobile phosphorus nutrient. The concentration of H^+ ions also influences the decrease in soil pH values. If the concentration of H^+ ions in the soil solution increases, the soil pH value will decrease. In addition, the application of organic fertilizer in the decomposition process is thought to produce organic acids, carboxylic acids, and phenolic acid groups as weak acids that release H^+ ions, so it is thought to decrease soil pH (Havlin et al., 2017).

Plant Growth of *E. palmifolia*

The ANOVA results in Table 4 show that applying organic fertilizer resulted in a Dayak onion plant height that was no different from treatment without organic fertilizer at plant ages of 4 to 12 WAP (P > 0.05). The increase in plant height from 10 to 12 WAP was 3 - 9 cm with a plant height range of 51 - 54 cm at the end of the observation. The control treatment experienced a decrease at the age of 10 WAP compared to the age of 8 WAP because it had a different number of plants. The number of plants at the age of 10 WAP increased compared to the age of 8 WAP because in general, the growth of *E. palmifolia* was not uniform and experienced slow growth. The same thing also happened to the leaf number variable (Table 5). The application of different types of organic fertilizer did not significantly affect the number of leaves of *E. palmifolia* plants at the age of 4 to 12 WAP (P > 0.05). The number of leaves at 6 WAP in the complete fertilization treatment (cow manure, guano, and rice husk ash) was more significant than without organic fertilization. Still, it was similar to other organic fertilizer treatments.

Table 4
Response of plant height of E. palmifolia *with the application of different organic fertilizers*

Treatment	Plant Height (cm)*				
	4	6	8	10	12
	WAP (Week After Planting)				
Without fertilization	14.3a	34.5a	45.8a	44.6a	52.6a
Cow Manure + Guano + Rice Husk Ash	15.7a	30.4a	41.4a	48.2a	50.5a
Cow Manure + Guano	17.1a	28.6a	42.5a	48.3a	52.6a
Guano + Rice Husk Ash	14.2a	28.0a	41.1a	47.8a	51.0a
Cow Manure + Rice Husk Ash	16.1a	29.7a	44.0a	45.2a	53.6a

Note: The numbers in the same column followed by the same letter show that they are not significantly different from the DMRT (*Duncan Multiple Range Test*) test at the significance level α = 5% (P > 0.05).
*: Transformation result = sqrt(x)

Plant height and number of leaves are not different between organic fertilizer treatment and control (without organic fertilizer), which can be expected because the nutrient levels in the soil or experimental field are classified as medium (N-total), high (C-organic), and very high (phosphorus and potassium). This allows plants to be able to take up or absorb nutrients that are already available in the soil. The application of organic fertilizer (cow manure, guano, and rice husk ash), which is slow release, still requires a longer time for

Table 5
Response of the number of leaves of E. palmifolia *with the application of different organic fertilizers*

Treatment	**Number of Leaves***				
	4	**6**	**8**	**10**	**12**
	WAP (Week After Planting)				
Without fertilization	2.7a	7.3a	13.5a	15.8a	21.1a
Cow Manure + Guano + Rice Husk Ash	3.9a	11.1a	18.4a	27.1a	32.6a
Cow Manure + Guano	2.6a	8.4a	13.8a	19.0a	24.2a
Guano + Rice Husk Ash	2.8a	8.9a	14.9a	19.2a	26.7a
Cow Manure + Rice Husk Ash	2.6a	5.6a	10.9a	16.9a	21.3a

Note: The numbers in the same column followed by the same letter show that they are not significantly different from the DMRT (*Duncan Multiple Range Test*) test at the significance level α = 5% (P > 0.05).
*: Transformation result = sqrt(x)

the release of nutrients so that the effect of the application of organic fertilizer is also not visible until the plants are 12 WAP on the growth of plant height and number of leaves. According to Sari et al. (2020), the slow-release application of PUKAP JESTRO SR organic fertilizer is better than conventional fertilizers, single and compound fertilizers.

The increased number of leaves on *E. palmifolia* from 4 to 12 WAP results from the provision of organic fertilizer. Organic fertilizer contains complete macronutrients, one of which is nitrogen. Nitrogen is needed by plants in large quantities in the form of ammonium and nitrate. Nitrogen functions to form or synthesize protein and chlorophyll and stimulates plant vegetative growth. It is thought that the nutrients (N, P, and K) contained in organic fertilizer can be absorbed by plants (Minardi et al., 2020) and increase organic matter and soil structure (Yunilasari et al., 2020). This is supported by the results of the analysis of the N nutrient content in cow manure, which is by the Ministry of Agriculture Regulation Number 28/Permentan/SR.130/5/2009 so that it can increase the nutrients in the soil.

Plant Biomass Yield of *E. palmifolia*

Table 6 shows that organic fertilizer treatment didn't significantly affect the biomass components of *E. palmifolia* plants (P > 0.05). Application of complete organic fertilizer (cow manure + guano + rice husk ash) resulted in higher shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight than without organic fertilization. Complete organic fertilization provided shoot and root dry weights, respectively, 2.6 and 5.8 times higher than without organic fertilization.

The number of leaves was also accompanied by increased plant biomass weight, especially the wet and dry weight of the *E. palmifolia* plant canopy. The more leaves there are, the greater the plant's ability to carry out photosynthesis. The increased rate of assimilation or photosynthesis processes increases plant biomass weight. Photosynthate

Table 6
Response of the plant biomass component per plot of E. palmifolia *with the application of different organic fertilizers*

Treatment	**Fresh Shoot Weight* (g)**	**Fresh Root Weight* (g)**	**Dry Shoot Weight* (g)**	**Dry Root Weight* (g)**
Without fertilization	52.6a	3.6a	14.6a	0.6a
Cow Manure + Guano + Rice Husk Ash	151.2a	21.6a	37.9a	3.5a
Cow Manure + Guano	63.6a	10.4a	15.9a	1.9a
Guano + Rice Husk Ash	48.0a	11.4a	13.8a	2.1a
Cow Manure + Rice Husk Ash	51.2a	4.4a	14.5a	0.8a

Note: The numbers in the same column followed by the same letter show that they are not significantly different from the DMRT (*Duncan Multiple Range Test*) test at the significance level α = 5% (P > 0.05).
*: Transformation result = sqrt(x)

will be used for the formation of cell organelles and enzymes and as a substrate in the respiration process for plant growth and development

Providing complete organic fertilizer (cow manure + guano + rice husk ash) can increase the availability of nutrients in the soil and improve the fertility of the soil's physical, chemical, and biological properties. Jenira et al. (2018) stated that fertilizer derived from cow dung can improve soil fertility and lead to a sustainable agricultural system. Organic fertilizer can improve soil chemical properties (pH, available P, and P nutrient uptake) and black rice yields (Yuniarti et al., 2020). The application of additional fertilizer is needed to provide the required nutrients (Tustiyani et al., 2023). Nitrogen is an integral part of chlorophyll which acts as a light-capturing pigment in photosynthesis. The nutrient phosphorus plays a role in the source and transfer of energy, especially in the form of adenosine triphosphate (ATP). Phosphorus is also an important component in the synthesis of nucleic acids, coenzymes, nucleotides, phosphoproteins, phospholipids, and phosphate sugars. The nutrient potassium plays a role in osmotic pressure and ion balance and is involved in the synthesis and transport of photosynthesis products for production and storage in plants (seeds, fruit, and tubers) (Havlin et al., 2017).

Yield of *E. palmifolia*

The ANOVA results in Table 7 show that the application of organic fertilizer did not significantly affect the yield of *E. palmifolia* (P > 0.05). Overall, the application of complete organic fertilizer (cow manure, guano, and rice husk ash) resulted in higher bulb weight per plant and bulb weight per plant plot compared to without organic fertilization and the other organic fertilizer treatments. Providing complete organic fertilizer (cow manure, guano, and husk ash) resulted in bulb weight per plant plot 2.6 times higher than without organic fertilization.

Table 7
Response of the weight of the tuber per plant and per plot of E. palmifolia *with the application of different organic fertilizers*

Treatment	**Tuber Weight per Plant* (g)**	**Tuber Weight per Plot* (g)**
Without fertilization	9.9a	35.2a
Cow Manure + Guano + Rice Husk Ash	21.5a	89.8a
Cow Manure + Guano	10.1a	39.2a
Guano + Rice Husk Ash	11.1a	39.0a
Cow Manure + Rice Husk Ash	10.3a	42.2a

Note: The numbers in the same column followed by the same letter show that they are not significantly different from the DMRT (*Duncan Multiple Range Test*) at the significance level α = 5% (P > 0.05). *: Transformation result = sqrt(x)

The bulb part of the *E. palmifolia* plant is an organ for storing food reserves. The bulb organ is also part of the sink, which requires photosynthesis to be transported by the leaves in the photosynthesis process. Increasing the rate of photosynthesis is also thought to increase plant weight or yield in the form of bulb weight per plant and plot. Complete fertilization (cow manure + guano + rice husk ash) can increase soil fertility and the availability of nutrients in the soil. This is in line with the research results (Ali et al., 2018) that the increase in the weight of onion bulbs is thought to be due to the role of nutrients, which support the enzyme activation process, protein synthesis, chlorophyll formation, root growth, and cell division from the application of organic fertilizer. Similar results were previously reported by Marlin et al. (2022) that the combination of nitrogen (100 kg N ha^{-1}) and kalium (25 kg K_2O ha^{-1}) can produce the highest number of bulbs and fresh bulb weight of *E. palmifolia*. Wiendi et al. (2021) also state that the application of NPK fertilizer increases the yield of *E. bulbosa* in various doses.

The bulb weight per *E. palmifolia* plant produced in this study was also lower than the results of the research from Atikah et al. (2021) with organic fertilizer and NPK treatment, namely 62.9 g $plant^{-1}$. This is likely because the shading conditions of the coffee plants cause the low light received by *E. palmifolia*. *E. palmifolia* requires sufficient sunlight in open conditions with lighting of ± 70% to assist in the formation of bulbs as a food reserve from photosynthesis.

Flavonoid Content of *E. palmifolia*

Anova's results showed that different organic fertilizer treatments had no significant effect on the flavonoid content of *E. palmifolia* bulbs (P > 0.05) (Table 8). Application of organic fertilizer with different combinations produces higher flavonoid levels compared to those without organic fertilizer. The increase in flavonoid content is because organic fertilizer treatment can increase the number of soil microbes, making it possible to increase interactions between plant roots and soil microbes. The interaction between microbes and plant roots is thought to increase the formation of chemical components, including flavonoids, which have high antioxidant activity (Shabira et al., 2022). Based on the research of Hamad et al. (2023) and Naguib et al. (2012), the giving of organic fertilizer can increase flavonoid content in the *Moringa oleifera* leaves and broccoli, respectively. The combination of organic and inorganic fertilizer treatments affects flavonoids and catechins contents in tea quality by regulating the phenylpropanoid and flavonoid biosynthesis pathways involved in the modulation of structural genes, such as *PAL* (*phenylalanine ammonia-lyase*) (Raza et al., 2024).

Table 8
Response of the flavonoid content of E. palmifolia *with the application of different organic fertilizers*

Treatment	Flavonoid Content (%)
Without fertilization	0.159a
Cow Manure + Guano + Rice Husk Ash	0.185a
Cow Manure + Guano	0.209a
Guano + Rice Husk Ash	0.199a
Cow Manure + Rice Husk Ash	0.209a

Note: The numbers in the same column followed by the same letter show that they are not significantly different from the DMRT (*Duncan Multiple Range Test*) at the significant level $\alpha = 5\%$ ($P > 0.05$)

Apart from that, it is thought that the intercropping planting pattern between coffee plants and *E. palmifolia* can also influence flavonoid levels through interactions between *Rhizobium* sp. bacteria and arbuscular mycorrhizal fungi, and plant growth can stimulate rhizobacteria and nematodes. This intercropping planting pattern can increase the supply of organic material, which can increase the number of soil microbes, such as bacteria, fungi, and nematodes, which are useful for the growth of the roots of *E. palmifolia* plants (Rostaei et al., 2018; Salehi et al., 2019; Sutrisno & Yusnawan, 2018). Besides that, light stress from the canopy of coffee trees increases flavonoid content because the enzymes of *PAL* also increase in the phenylpropanoid pathway (Firdaus et al., 2022).

Microclimates Observation

Apart from observing the growth of *E. palmifolia* plants, air temperature, light intensity, and shade percentage were also measured (Table 9). The average air temperature under the coffee trees at five (5) observations, namely from 4 to 12 WAP, was around 29.7°C. The results of air temperature measurements are the results of air temperature observations from the Climatology station in Figure 1a which show that the temperature range is still suitable for the growth of *E. palmifolia*. The decrease in temperature is followed by increasing in air humidity in Figure 1b which shows the range of 82 – 85% which is still good for the growth of *E. palmifolia*. Purwanti and Taryono (2018) state that *E. palmifolia* can still grow with an air humidity range of 30 – 70%.

Table 9
Results of measurements of air temperature, sunlight intensity, and percentage of shading

Time Measurement (WAP)	The Average Air Temperature Under the Canopy of Coffee Tree (°C)	The Average of Light Intensity (Lux)		Percentage of Shade (%)
		Under the Coffee Tree	Outside of the Coffee Tree	
4	30.5	1652.2	3743.2	55.86
6	30.5	661.2	157954.6	99.58
8	28.6	527.4	46641.4	98.87
10	30.5	662.0	61402.8	98.92
12	28.2	539.0	46092.0	98.83
Average	29.7	808.36	63166.8	90.41

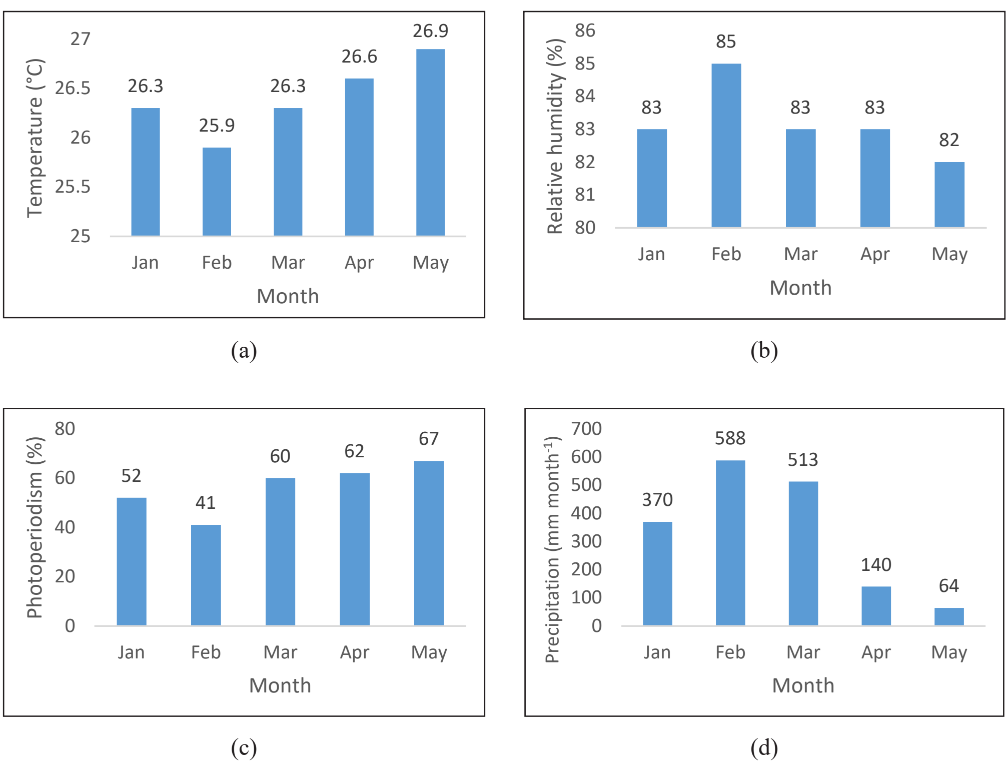

Figure 1. The observation of climate elements during research from the Sleman Regency Climatology station. Temperature (a), relative humidity (b), photoperiodism (c), and precipitation (d)

The average light intensity, both under the coffee tree and outside the coffee tree area, shows varying results. The average intensity of sunlight under coffee trees is lower than outside the coffee tree area. The average percentage of shade in observations from 4 to 12 WAP shows around 90.41%. These shade conditions cause low light intensity to be received by *E. palmifolia*. *E. palmifolia* requires sufficient photoperiodism in open conditions with the lighting of ± 70%, meanwhile the results of observing the highest photoperiodism in Figure 1c show a result of 67% which is still by the photoperiodism requirement for the growth of *E. palmifolia*.

The research location, which is located in Umbulharjo Village, Cangkringan District, has an area of approximately ± 103 ha with topography in the form of hills and lowlands at an altitude of ± 662 m above sea level and is ± 12,5 km from the peak of Mount Merapi. The type of coffee cultivated is Robusta which has growing requirements at an altitude of 100 – 600 m above sea level, rainfall of 1250 – 2500 mm/year or < 60 mm/month, and air temperature of 21 – 24°C (Kementerian Pertanian Republik Indonesia, 2014). Based on data from Climatology Station shows the highest precipitation was 588 mm occurred in February 2023, while the lowest precipitation occurred in May 2023 at 64 mm (Figure 1d) and the average maximum precipitation is 64.8 mm/month (Statistics Sleman Regency, 2021). This shows that *E. palmifolia* plants can still grow and produce in high precipitation with highland topography, although the best results are obtained from lowland topography with air temperatures between 25 - 32°C and dry climates.

CONCLUSION

The application of complete organic fertilizer (cow manure + guano + rice husk ash) produces a higher number of leaves, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, tuber weight per plant (21.5 g), and tuber weight per plot (89.8 g) compared to without fertilization. The application of organic fertilizer produces the flavonoid content of *E. palmifolia* bulbs 1.2 – 1.3 times higher than no fertilization. The average percentage of shade obtained was 90.41% with an average air temperature of around 29.7°C. *E. palmifolia* can be planted under the coffee tree as an alley cropping. Based on the characteristics of the growth and yield of *E. palmifolia*, further research on the farming feasibility analysis needs to be considered.

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Effect of Different Pasteurization Temperatures on the Physicochemical and Microbiological Quality of Rock Melon Juice During Storage at 4°C

Siew Hui Lee, Syamilah Nordin and Norlia Mahror*

Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia

ABSTRACT

Fruit juice, a popular beverage, is prone to spoilage due to its high moisture and nutritional content, which may pose health risks to consumers. This study aims to evaluate the changes in physicochemical and microbiological qualities of rock melon juice (RMJ) subjected to two pasteurization methods: mild-temperature-short-time (MTST) pasteurization at 70°C for 30 s and high-temperature-short-time (HTST) pasteurization at 85°C for 20 s. The juice was stored at 4°C for 21 days, with total soluble solids (TSS), titratable acidity (TA), pH, TSS/TA ratio, colour, total plate count (TPC), and yeast and mold counts assessed on days 1, 7, 14, and 21. Results indicated that pasteurization significantly affected ($p < 0.05$) the physicochemical and microbiological qualities of RMJ, except for TSS, TA, and pH. On day 1, both treatments increased the L* value (control: 68.16, MTST: 69.50, HTST: 69.70) while reducing a* (control: 20.15, MTST: 16.84, HTST: 15.87) and b* (control: 51.14, MTST: 46.67, HTST: 46.96) values, resulting in a lighter juice colour due to pigment degradation. MTST and HTST samples showed lower TPC (2.15 and 2.00 log CFU/mL) compared to the control sample (3.96 log CFU/mL) on day 1. During 21 days of storage, the microbial loads of pasteurized RMJ were observed at 2 log CFU/mL and lower. No significant differences ($p > 0.05$) were observed in TSS, TA, and microbiological quality between MTST- and HTST-treated RMJ after storage. HTST pasteurization is recommended to minimize colour changes, potentially enhancing juice acceptability and marketability.

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E-mail addresses:
mivislee@gmail.com (Siew Hui Lee)
syamilahnordin@gmail.com (Syamilah Nordin)
norlia.mahror@usm.my (Norlia Mahror)
*Corresponding author

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INTRODUCTION

Rock melon (*Cucumis melo* var. *reticulatus*) is a warm-season cucurbit species known for its distinctive spherical or oval shape,

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characterized by a rough, raised netting pattern covering its skin. Its orange flesh is filled with seeds. This delicious and nutritious fruit is enjoyed worldwide for its sweet and aromatic flavour and its phytonutrient content. It is highly beneficial for health since it offers high nutrition, antioxidants, phenolic compounds, ascorbic acid, carotenoids, essential vitamins, and minerals (Hassan et al., 2022). Rock melon is well-known for its juiciness and sweetness, and it is commonly enjoyed fresh as a healthy dessert or blended into refreshing juices. According to the Department of Agriculture (DOA) Malaysia, the cultivation of rock melons is primarily located in the states of Kedah, Kelantan, Pahang, and Sabah, with a total harvested area of 283.48 ha.

However, the short shelf life of rock melon juice (RMJ) is a significant drawback due to its high water content, which makes the juice vulnerable to microorganisms and degradation (Mandha et al., 2023). The untreated RMJ provides a conducive environment for microbial growth and supports metabolic activities due to its natural sugars, which serve as a source of nutrients for the microorganisms. This poses a critical risk to public health, as the consumption of juices contaminated with foodborne pathogens, such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, or *Salmonella* spp., can result in severe illness. Numerous foodborne outbreaks linked to pathogen-contaminated fruit juices have been reported globally. Furthermore, the degradation of untreated juice could lead to changes in its physicochemical properties, such as turbidity, colour and taste, which can adversely affect its overall quality (Salin et al., 2022). The physicochemical changes in fruit juices are primarily driven by enzymatic activity, which accelerates chemical reactions and results in the degradation of organic compounds. One key enzyme involved in this process is polyphenol oxidase (PPO), which is responsible for the browning of juices and unfavourably impacting their colour. These challenges underscore the necessity of implementing effective preservation methods to extend the shelf life of RMJ, ensuring microbial safety, and maintaining quality for consumer acceptance.

The quality and safety of fruit juices, including RMJ, are key concerns for consumers and the food industry. Thermal pasteurization is a common preservation technique used in the food industry. Pasteurization is a heat treatment to kill pathogens and reduce the number of spoilage microorganisms (Amine et al., 2023). The United States Food and Drug Administration (USFDA) implies a 5-log reduction of the most resistant microorganisms of public health concern that may occur in juices (United States Food and Drug Administration [USFDA], 2004). This will extend the juice shelf life due to reduced spoilage organisms. This method also helps inactivate enzyme activity that could lead to undesirable sensory and nutritive changes and affect the juice viscosity and off-flavour formation (Ongaratto & Viotto, 2016; Taranto et al., 2017).

However, thermal pasteurization comes with inherent drawbacks to the fruit juice. One primary concern is its effect on the colour, taste, and flavour of the final product, the consequence of pigment and flavour compound degradation during heat treatment. The heat treatment also leads to the degradation of bioactive compounds, including carotenoids and phenolic compounds (Ferreira et al., 2022). Therefore, understanding the changes in the physicochemical properties of RMJ is crucial for both the industry and consumers. Several pasteurization techniques are widely used in the food industry, such as high-temperature-short-time (HTST), mild-temperature-short-time (MTST), and low-temperature-long-time (LTLT).

It is also necessary to optimize the pasteurization temperature and duration to achieve optimal conditions that minimize quality changes in the juice (Mandha et al., 2023). For example, Vegara et al. (2013) suggested that the combination of low temperature and short pasteurization time (65°C for 30 s) of pomegranate juice can effectively inactivate microorganisms with minimal changes in their quality. Moreover, the storage duration of juice also influences the quality of juice, such as colour degradation due to residual enzyme activity during the thermal process and microbial stability due to microbial growth over time, which can compromise the product's safety. Marie-Michel et al. (2020) and Queirós et al. (2015) demonstrated that pasteurization conditions at 70°C for 30 s and 82°C for 20 s sufficiently eliminate microorganisms in cherry and watermelon juice during refrigeration storage for several weeks. Besides, the industrial thermal pasteurization conditions for premium juices are usually set at 71.1°C for 30 s, 90°C for 2 s, or 84°C for 20 s (Queirós et al., 2015). However, the effects of pasteurization on the physicochemical and microbiological properties of RMJ have not been extensively studied, leaving a gap in understanding how these treatments influence its storage stability as well as physicochemical and microbiological quality. Hence, the present study aims to evaluate the effect of MTST (70°C for 30 s) and HTST (85°C for 20 s) pasteurization on the physicochemical and microbiological quality of RMJ during storage at 4°C.

MATERIALS AND METHODS

Preparation of Rock Melon Juice (RMJ)

Ripe rock melon fruits were purchased from AEON Queensbay Mall Pulau Pinang, Malaysia, and immediately transported to the food processing laboratory at Universiti Sains Malaysia. The preparation of RMJ was carried out in several stages. Rock melon fruits were washed thoroughly under running water to remove any dirt or contaminants from the surface. Then, the rock melon fruits were cut in half crosswise, and the seeds were removed and discarded. The skin covering the fruits was slid off using a kitchen knife, leaving the orange-coloured flesh. The halved melons were cut into smaller pieces and transferred into a fruit juice extractor. The juice was collected, and the fruit pulp was discarded. No preservatives, sugar, or water were added to prepare a healthy juice.

Pasteurization of RMJ

In this experiment, two different pasteurization conditions were used: MTST (70°C for 30 s) and HTST (85°C for 20 s). In a warm water bath, RMJ was pasteurized in sterilized beakers covered with aluminium foil. The temperature of the juice at the centre of the tube was regularly monitored using a thermometer. The treatment time was measured after the juice samples reached the target temperature. Then, the RMJ was quickly cooled in an ice-water bath. The control was unpasteurized RMJ. The samples were then stored in 100 mL polyethylene terephthalate (PET) bottles, and the bottles were tightly closed with screw caps. The following terms were used to describe the different treatments in this study: control (freshly squeezed juice), MTST (pasteurization at 70°C for 30 s), and HTST (pasteurization at 85°C for 20 s). All treatments and analyses were conducted in duplicate. The microbiological and physicochemical parameters of the RMJs were analysed on the 1st, 7th, 14th, and 21st days of storage at 4°C.

Physicochemical Analysis

Determination of pH

The pH of RMJ was determined using a pH meter (Mettler Toledo Delta 320, China) at room temperature. The unpasteurized and pasteurized RMJ samples were placed in separate beakers, and the pH meter probe was inserted into the samples.

Determination of Titratable Acidity (TA)

The TA of RMJ was expressed as citric acid content per unit volume (Marie-Michel et al., 2020). An amount of 5 mL RMJ sample was added to 50 mL of distilled water. The TA was determined by volumetric neutralization with 0.1 M sodium hydroxide solution, and phenolphthalein was used as the indicator. The acidity in the sample was obtained by considering the equivalent weight of citric acid, which is equal to 64.04 g/mol, according to the following formula [Equation 1] :

$$TA(\%) = \frac{V_{NaOH} \times N_{NaOH} \times 64.04}{V_{RMJ} \times 1000} \times 100 \qquad [1]$$

Determination of Total Soluble Solid (TSS). TSS was determined by dropping a few drops of RMJ on the prism of a Labart-90 copper refractometer with automatic temperature compensation (ATC, China) in terms of °Brix.

Colour Measurement. The colour measurement of the RMJ sample was determined using the Konica Minolta Spectrophotometer CM-3500d (Japan). The colour values were expressed as L* (whiteness or brightness/darkness), a* (redness/greenness), and b* (yellowness/blueness) at any time. The colour parameters were read thrice for each sample. Hue angle (h°), chroma (C*), and total colour difference (ΔE) were calculated from L*, a*, and b* values, using the formulas below [Equation 2-4]:

$$h° = tan^{-1} \frac{b^*}{a^*} \quad [2]$$

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

$$\Delta E^* = \sqrt{(L_1^* - L_2^*)^2 + (a_1^* - a_2^*)^2 + (b_1^* - b_2^*)^2} \quad [4]$$

Microbiological Analysis

A volume of 1 mL RMJ sample was taken aseptically and transferred to 9 mL buffered peptone water (BPW) in a sterile dilution bottle. The solution was mixed thoroughly to create a 10^{-1} dilution. Then, 1 mL solution from the 10^{-1} dilution bottle was mixed with 9 mL BPW in a new sterile dilution bottle. This created a 10^{-2} dilution. The processes were repeated to create dilution factors of 10^{-3}, 10^{-4}, and 10^{-5}. An amount of 0.1 mL aliquots of each dilution was dispensed on plate count agar (PCA) and dichloran rose bengal chloramphenicol (DRBC) agar using the standard spread plate method. PCA was used to enumerate the total plate count, while DRBC agar was used for yeast and mold. The agar plates were incubated at a specific temperature: PCA was incubated at 37°C for 48 hours, and DRBC agar was incubated at 30°C for 7 days. After incubation, the visible colonies on each plate were counted. Counts of visible colonies were expressed as colony forming units per mL (CFU/mL) of sample and calculated using the formula below (Equation 5) :

$$CFU/mL = \frac{Number\ of\ colonies\ per\ plate\ \times\ dilution\ factor}{Volume\ of\ sample\ plated\ (mL)} \quad [5]$$

Statistical Analysis

Data obtained were subjected to statistical analysis using Statistical Package for the Social Sciences (SPSS) 28.0 software (SPSS Inc., IBM). In this study, data were represented as mean values ± standard deviation (SD; $n = 2$). The significant differences between mean values of juice samples were determined by analysis of variance (one-way ANOVA) using Tukey's HSD (Honestly Significant Difference) test at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Effect of Different Pasteurization Temperatures on the Physicochemical Quality of RMJ

The changes in pH, TA, and TSS in pasteurized and unpasteurized RMJ samples were examined over a 21-day storage period, as shown in Table 1. No significant difference was observed between the pasteurized and unpasteurized RMJ in terms of pH, TA, and TSS. This suggests that the thermal process did not significantly affect these parameters. Nevertheless, a significant difference in pH and TA values between unpasteurized and pasteurized samples was shown on days 14 and 21 of storage.

Table 1
Physicochemical quality of the pasteurized and unpasteurized RMJ samples

Parameter	Treatment	Storage period (day)			
		1	7	14	21
pH	Control	6.80 ± 0.04^{Aa}	6.90 ± 0.05^{Aa}	5.61 ± 0.28^{Ba}	5.20 ± 0.04^{Ba}
	MTST	6.73 ± 0.01^{Aa}	6.88 ± 0.11^{Aa}	6.92 ± 0.16^{Ab}	6.95 ± 0.06^{Ab}
	HTST	6.71 ± 0.02^{Aa}	6.97 ± 0.23^{ABa}	7.25 ± 0.04^{ABb}	7.37 ± 0.16^{Bb}
TA (%)	Control	0.12 ± 0.01^{Aa}	0.09 ± 0.00^{Aa}	0.21 ± 0.01^{Aa}	0.39 ± 0.08^{Ba}
	MTST	0.10 ± 0.00^{Aa}	0.10 ± 0.0^{Aa}	0.11 ± 0.01^{Ab}	0.10 ± 0.03^{Ab}
	HTST	0.10 ± 0.01^{Aa}	0.09 ± 0.02^{Aa}	0.10 ± 0.01^{Ab}	0.11 ± 0.01^{Ab}
TSS (°Brix)	Control	8.00 ± 0.00^{ABa}	7.75 ± 0.35^{Aa}	7.75 ± 0.35^{Aa}	8.25 ± 0.35^{ABa}
	MTST	7.75 ± 0.35^{Aa}	8.25 ± 0.35^{Aa}	7.50 ± 0.71^{Aa}	8.00 ± 0.00^{Aa}
	HTST	7.75 ± 0.35^{Aa}	8.25 ± 1.06^{Aa}	8.50 ± 0.71^{Aa}	7.75 ± 0.35^{Aa}
TSS/TA ratio	Control	67.14 ± 7.91^{Aa}	86.11 ± 3.93^{Aa}	37.05 ± 4.18^{Ba}	21.57 ± 3.78^{Ba}
	MTST	77.50 ± 3.53^{Aa}	82.50 ± 3.53^{Aa}	68.34 ± 2.35^{Ab}	83.33 ± 23.57^{Aa}
	HTST	81.95 ± 9.82^{Aa}	98.57 ± 12.12^{Aa}	89.45 ± 0.78^{Ac}	71.25 ± 12.37^{Aa}

Note: Results are expressed as means ± standard deviations from a duplicate samples. Means values with the same superscript uppercase letter within the same row indicate no significant difference by storage time ($p > 0.05$). Mean values with the same superscript lowercase letter within the same column indicate no significant difference by processing method ($p > 0.05$). Control (unpasteurized RMJ); MTST (pasteurization at 70°C for 30 s); HTST (pasteurization at 85°C for 20 s). Abbreviations: TSS, total soluble solid; TA, titratable acidity

pH

The pH of pasteurized samples increased towards neutral, while the unpasteurized sample decreased and became more acidic by day 14. The pH of unpasteurized RMJ decreased significantly from 6.80 to 5.20 after cold storage, which was similar to some studies (Ferreira et al., 2022; Lagnika et al., 2017; Techakanon & Sirimuangmoon, 2020). The decreased pH could be due to the lactic acid production by the lactic acid bacteria, which also reflected the increase of bacterial counts in the unpasteurized sample during storage.

Another reason for the decrease in pH is the dissociation of citric acid from the fruits (Kong et al., 2020). A slight increase in pH was found in MTST and HTST samples after 21 days. The increased pH trend was also observed in sweet cherry juice, as reported by Queirós et al. (2015). The increase in pH was attributed to the acid hydrolysis of some polysaccharides into disaccharides like starch into sucrose, fructose, and glucose, which is believed to have altered the taste of fruit juice by making it sweeter and less sour (Rehman et al., 2014). There were no significant changes in pH between the MTST and HTST samples, which aligns with the findings by Kong et al. (2020) and Vegara et al. (2013). This stability might be due to the pasteurization temperatures in MTST and HTST that were not high enough to cause significant degradation of citric acid, which has a decomposition temperature of 165°C (Tsioptsias et al., 2024). Additionally, the buffering capacity of RMJ may have contributed to maintaining its pH despite thermal treatment.

TA

The TA of unpasteurized RMJ significantly increased after 21 days as the pH decreased. The TA of unpasteurized RMJ significantly increased after 21 days, corresponding with a decrease in pH. A significant difference in TA was observed between pasteurized and unpasteurized RMJ during days 14 to 21 of storage, paralleling the changes in pH. The increase in TA for the unpasteurized RMJ can be attributed to microbial activity, particularly the fermentation of sugars by naturally occurring microorganisms, leading to the production of organic acids such as lactic and acetic acids. The accumulation of these acids contributes to higher TA values and a lower pH. In contrast, pasteurization inactivates most spoilage microbes, slowing acid production and preserving juice stability over time (Ağçam et al., 2018; Mandha et al., 2023).

TSS

No significant changes in TSS were observed in both pasteurized samples, while a slight decrease in TSS was recorded in the unpasteurized sample. This is because intense pasteurization condition effectively inhibits yeast growth, preventing further changes (Techakanon & Sirimuangmoon, 2020). In pasteurized RMJ, TSS is expected to remain relatively constant since microbial activity is inhibited, preventing sugar breakdown. However, in unpasteurized RMJ, TSS may decrease due to microbial fermentation, where sugars are metabolized into organic acids and other byproducts.

TSS/TA Ratio

The increase of TA in control samples over time resulted in a decrease in the TSS/TA ratio, from 67.14 to 21.57. The decrease in TSS/TA ratio influenced the flavour profile of RMJ as the acidity became stronger (Roongruangsri et al., 2013). The MTST sample was predicted to be slightly sweeter than the HTST on day 21 because the TSS/TA ratio in MTST was higher.

The TSS, TA, and pH of unpasteurized RMJ were 8.00 °Brix, 0.122%, and 6.80, respectively. The TSS of unpasteurized RMJ was similar to many juice products, which has the range between 5 to 16 °Brix (Babarinde et al., 2019; Basak et al., 2022; Kasim & Kasim, 2014; Mandha et al., 2023). The variation depends on the fruit variety, growth condition, maturity, and climate (Lazano, 2006). TSS in the RMJ mainly contains sugars, acids, and other nutritional compounds, such as vitamins and minerals. The pH value of unpasteurized RMJ was typically higher than other fruits, such as orange juice (pH 2.90; Azzouzi et al., 2018), pineapple juice (pH 4.10; Lagnika et al., 2017), sweet cherry juice (pH 3.68; Queirós et al., 2015), mango juice (pH 4.83; Babarinde et al., 2019), and passion fruit (pH 3.39; Kaddumukasa et al., 2017). The finding indicates that RMJ is generally sweeter and less tart than other fruit juices.

According to the Codex Alimentarius, the minimum °Brix value of melon juice is 8.0 °Brix. In this study, the TA of RMJ ranged from 0.09% to 0.39%, while TSS was between 7.50 to 8.50 °Brix. The slightly lower value of TSS could be due to the absence of additives such as sugars to increase the solid content in the juice. Therefore, the incorporation of additives to enhance solid content may be explored to meet the specified criteria and to ensure compliance with established quality benchmarks for fruit juice products.

Colour

Table 2 shows the colour changes in pasteurized and unpasteurized RMJ during storage. The value L* represents the lightness, a* depicts the redness, and b* indicates the yellowness of the juice. The RMJ juice is a light-coloured juice, i.e., dominant with a yellow-orange colour.

All colour components of pasteurized RMJ significantly change compared to the unpasteurized RMJ, indicating that the thermal process can significantly affect the colour of juice. Pasteurized RMJ had higher L* values but lower a* and b* values than unpasteurized RMJ, which resulted in a lighter colour and the loss of the red and yellow colours. Lee and Coates (1999) also reported a similar observation of slight increases in L* value after pasteurization in grapefruit juices, which was attributed to partial precipitation of unstable and suspended particles in the juices. Carotenoids are the pigments in rock melon responsible for the orange colour of the flesh. However, carotenoids are unstable when exposed to heat and light; hence, degradation occurs during thermal pasteurization, leading to the reduction of the red and yellow colours (Sharma et al., 2008). The isomerization of carotenoids at high temperatures also contributed to the colour changes (Santhirasegaram et al., 2013).

Table 2
Colour changes of pasteurized and unpasteurized RMJ samples

Colour components	Treatment	Storage period (day)			
		1	7	14	21
L*	Control	68.16 ± 0.23^{Aa}	65.19 ± 0.05^{Ba}	59.98 ± 0.83^{Ca}	57.23 ± 0.09^{Da}
	MTST	69.50 ± 0.13^{ABb}	68.54 ± 1.09^{Ab}	68.97 ± 2.31^{Ab}	74.73 ± 0.38^{Bb}
	HTST	69.70 ± 0.06^{Ab}	70.17 ± 0.83^{Ab}	70.75 ± 1.66^{Ab}	73.27 ± 0.08^{Ac}
a*	Control	20.15 ± 0.37^{Aa}	19.34 ± 0.14^{Aa}	19.03 ± 0.30^{Aa}	19.40 ± 0.30^{Aa}
	MTST	16.84 ± 0.10^{Ab}	17.08 ± 0.30^{Ab}	15.52 ± 1.33^{Aab}	10.33 ± 0.32^{Bb}
	HTST	15.87 ± 0.82^{Ab}	14.79 ± 0.16^{ABc}	13.62 ± 1.41^{ABa}	11.68 ± 0.02^{Bc}
b*	Control	51.14 ± 0.69^{Aa}	54.72 ± 0.03^{Aa}	54.96 ± 0.53^{Aa}	53.24 ± 2.65^{Aa}
	MTST	46.67 ± 0.01^{Ab}	46.96 ± 0.29^{Ab}	41.06 ± 1.61^{Bb}	32.31 ± 0.8^{Cb}
	HTST	46.96 ± 0.02^{Ab}	48.96 ± 0.81^{Ab}	49.06 ± 1.09^{Ac}	48.12 ± 0.13^{Aa}
Hue angle (°)	Control	68.50 ± 0.10^{Aa}	70.53 ± 0.12^{Aa}	70.91 ± 0.11^{Aab}	69.96 ± 1.20^{Aa}
	MTST	70.16 ± 0.11^{ABab}	70.01 ± 0.21^{ABa}	69.32 ± 0.88^{Aa}	72.27 ± 0.93^{Ba}
	HTST	71.33 ± 0.91^{Ab}	73.20 ± 0.09^{Ab}	74.51 ± 1.20^{Bb}	76.36 ± 0.01^{Bb}
Chroma	Control	54.97 ± 0.78^{Aa}	58.04 ± 0.07^{Aa}	58.16 ± 0.60^{Aa}	56.67 ± 2.38^{Aa}
	MTST	49.61 ± 0.03^{Ab}	49.96 ± 0.35^{Ab}	43.90 ± 1.98^{Bb}	33.93 ± 0.67^{Cb}
	HTST	49.57 ± 0.24^{Ab}	51.14 ± 0.82^{Ab}	50.92 ± 1.43^{Ac}	49.52 ± 0.13^{Ac}
ΔE	Control	-	4.75 ± 0.63^{Aa}	9.10 ± 0.48^{Ba}	11.24 ± 0.28^{Ca}
	MTST	5.73 ± 0.73^{Aa}	5.28 ± 1.24^{Aa}	11.22 ± 1.36^{Ba}	22.24 ± 039^{Cb}
	HTST	6.18 ± 1.36^{Aa}	6.14 ± 0.06^{Aa}	7.41 ± 2.73^{Aa}	10.35 ± 0.53^{Aa}

Note: Results are expressed as means ± standard deviations from a duplicate sample. Mean values with the same superscript uppercase letter within the same row indicate no significant difference by storage time ($p > 0.05$). Mean values with the same superscript lowercase letter within the same column indicate no significant difference by processing method ($p > 0.05$). Control (unpasteurized RMJ); MTST (pasteurization at 70°C for 30 s); HTST (pasteurization at 85°C for 20 s)

On day 21 of storage, the L* values of MTST continued to increase significantly, from 69.50 to 74.72. MTST sample showed a significant difference from HTST, resulting in a lighter colour on day 21, as shown in Figure 1. The a* and b* values of MTST and HTST also showed a significant difference, in which MTST had a higher reduction of colour pigments than HTST. This situation could be due to the heating duration of MTST (30 s), i.e., longer than HTST (20 s), causing a more severe degradation. The b* value of HTST at day 21 of storage showed no significant changes throughout the storage period and no significant difference with the unpasteurized RMJ. Therefore, HTST resulted in better colour retention compared to MTST.

Meanwhile, significant changes in the colour components during storage were detected in the unpasteurized RMJ. The L* value of unpasteurized RMJ decreased significantly from 68.16 to 57.23 during the storage period, which led to a darker colour. Rabie et al. (2015) also reported a decrease in the L* value of untreated physalis juice. Likewise, Mandha et al. (2023) stated that the colour compounds in unpasteurized RMJ were unstable due to

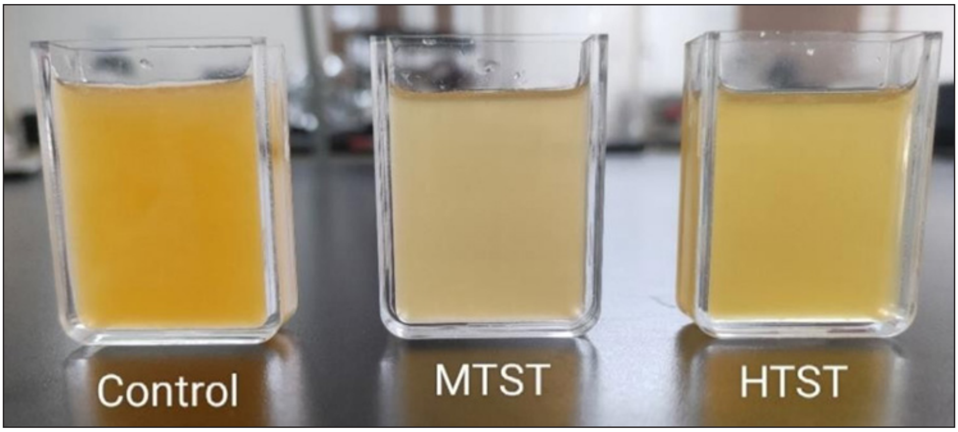

Figure 1. Colour of pasteurized and unpasteurized RMJ samples at day 21 of storage

residual enzymatic activities. However, HTST samples exhibited an increase in L* and b* values during the storage period. This could be due to enzymes like PPO and peroxidase (POD), which are responsible for the inactivation of the browning reactions during the thermal process.

A greater increase in hue angle was found in HTST samples compared to the MTST and control, indicating HTST samples had a slight shift toward a more yellowish appearance. Chroma, which represents colour intensity, maintained relatively higher values in the control samples (54.97 to 56.67), indicating stable saturation. MTST samples exhibited a significant chroma reduction (49.61 to 33.93), suggesting pigment breakdown. HTST samples showed minimal changes in chroma (49.57 to 49.52), demonstrating that shorter treatment time better preserves colour intensity. MTST samples showed the highest colour degradation, with ΔE reaching 22.24 on day 21, indicating significant visible colour loss. HTST samples had the lowest ΔE values, which confirmed superior colour retention compared to MTST.

Effect of Different Pasteurization Temperatures on the Microbiological Quality of RMJ

Table 3 shows the result of microbiological analysis for total plate count and yeasts and mold count in pasteurized and unpasteurized RMJ samples during storage. After pasteurization of RMJ, the MTST and HTST samples resulted in a significant decrease of microbial load for both counts, indicating that the pasteurization process had successfully inactivated the microorganisms to a lower level. This result is congruent with previous studies, which reported the reduction of microbial count in pasteurized juices, with yeast and mold counts below the detection limit (Hu et al., 2020; Kong et al., 2020; Mandha et al., 2023; Queirós et al., 2015). The decrease in microbial load was due to the heat that killed the microorganisms by denaturing their enzymes and disrupting their cell membrane, rendering them unable to survive or reproduce effectively.

There was a gradual increase in the total plate count of unpasteurized RMJ during the 21-day storage period from 3.96 to 8.59 log CFU/mL, which was considered spoiled as the total plate count exceeded 5 log CFU/mL. According to Ma et al. (2020), unpasteurized fruit juice should be consumed within 4 hours of preparation to ensure its safety and quality. The pasteurized samples were found to have a total plate count of below 2.00 log CFU/mL on day 21 of storage, indicating a significant difference from the unpasteurized samples.

Table 3
Microbiological quality of pasteurized and unpasteurized RMJ samples

Colony count (log CFU/mL)	**Treatment**	**Storage period (day)**			
		1	**7**	**14**	**21**
Total plate count	Control	3.96 ± 0.46^{Aa}	6.43 ± 0.04^{Ba}	8.47 ± 0.01^{Ca}	8.59 ± 0.23^{Ca}
	MTST	2.15 ± 0.21^{Ab}	2.69 ± 0.13^{Ab}	3.17 ± 0.45^{Ab}	$< 2.00^{Bb}$
	HTST	2.00 ± 0.00^{Ab}	2.60 ± 0.42^{Ab}	2.80 ± 0.71^{Ab}	2.00 ± 0.00^{Ac}
Yeasts & mold count	Control	3.18 ± 0.25^{Aa}	2.65 ± 0.07^{Aa}	2.15 ± 0.21^{ABa}	$< 2.00^{Ba}$
	MTST	$< 2.00^{Ab}$	$< 2.00^{Ab}$	$< 2.00^{Ab}$	$< 2.00^{Aa}$
	HTST	$< 2.00^{Ab}$	$< 2.00^{Ab}$	$< 2.00^{Ab}$	$< 2.00^{Aa}$

Note: Results are expressed as means ± standard deviations from a duplicate sample. Mean values with the same superscript uppercase letter within the same row indicate no significant difference by storage time ($p > 0.05$). Mean values with the same superscript lowercase letter within the same column indicate no significant difference by processing method ($p > 0.05$). Control (Unpasteurized RMJ); MTST (pasteurization at 70°C for 30 s); HTST (pasteurization at 85°C for 20

On the contrary, the result of yeast and mold counts in the unpasteurized RMJ showed a gradual decrease of microbial load as the storage period increased, from day 1 (3.18 log CFU/mL) to day 21 (below 2.00 log CFU/mL). This result contradicted some studies in which the yeast and mold counts in unpasteurized juice continuously increased during the storage period at 4°C (Hu et al., 2020; Huang et al., 2018; Juliet et al., 2020; Leneveu-Jenvrin et al., 2020; Mandha et al., 2023; Queirós et al., 2015; Techakanon & Sirimuangmoon, 2020; Yildiz et al., 2021). Since the growth condition of yeast and mold is between 10°C to 35°C, the decrease in yeast and mold counts might be due to the long refrigeration storage condition (4°C), which slows down and prevents their growth. The yeast and mold counts of pasteurized samples was less than 2.00 log CFU/mL and remained unchanged for the 21 days of storage.

The shelf life of the pasteurized RMJ was expected to be longer than 21 days. Santhirasegaram et al. (2015) also found that fruit juice undergoing thermal pasteurization has a shelf life exceeding 5 weeks with almost no microbial growth. Commercial fruit juice products typically have a shelf life of several weeks, depending on their packaging, concentration, and storage conditions (Ashurst, 2016). If the quality of RMJ samples in this study were observed for a longer period, HTST might have a longer shelf life than MTST.

CONCLUSIONS

In conclusion, thermal pasteurization and storage significantly affected the physicochemical and microbiological qualities of RMJ, except for the TSS, TA, and pH. All the physicochemical and microbiological parameters of unpasteurized RMJ showed a significant difference during the 21 days of cold storage. The TSS and TA significantly increased, while the pH decreased to become more acidic. The colour of the juice became darker. The total plate count increased while yeast and mold counts decreased with the storage period. There were significant differences between the colour and pH of the HTST (85°C for 20 s) and MTST (70°C for 30 s) juices but no significant difference in the microbiological quality. The HTST-treated juice had a significant increase in pH at day 21 of storage, while MTST-treated juice had no significant difference. Furthermore, MTST-treated juice resulted in a lighter colour than HTST. The HTST-treated RMJ was better at retaining the juice quality as it had a minor colour change compared to MTST-treated RMJ. The HTST pasteurization method is recommended for its minimal colour changes and comparable pH stability to MTST.

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Review Article

Herbicide Resistance in Malaysian Oil Palm Plantations: A Comprehensive Review of Current Challenges and Management Strategies

Sadat Elutola Babalola, Muhammad Saiful Ahmad-Hamdani and Norazua Zakaria*

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

ABSTRACT

Elaeis guineensis Jacq. (oil palm) is the principal export crop in Malaysia, accounting for a significant percentage of GDP. However, its production is hampered by various factors, in particular weeds. Weed control in Malaysian oil palm plantations predominantly relies on herbicides. The excessive herbicide use has driven the emergence of herbicide-resistant weed species in plantations. Repeated use of herbicides such as glyphosate, glufosinate-ammonium, metsulfuron-methyl, and paraquat has led to the prevalence of resistant weed biotypes. Herbicide resistance in weeds is a growing global concern, threatening food safety and security, agricultural sustainability, and the environment. While much of the focus has historically been on well-known resistant species, lesser-known weeds, particularly those infesting oil palms, also exhibit resistance, causing significant long-term impacts. However, research on these lesser-known species is limited, especially regarding their competitive effects, resistance status, and underlying resistance mechanisms. Focusing on these less-studied herbicide-resistant weed species is essential for effective crop management and the sustainability of oil palm cultivation. To address these challenges, this review explores the background of herbicide-resistant weeds in Malaysian oil palm plantations and examines integrated approaches for sustainable weed management. These strategies include cover cropping, targeted livestock grazing, and using herbicides in diverse modes of action. Integrating multiple management strategies, closely monitoring weed populations for early detection of resistance, and responding promptly

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E-mail addresses:
babalolasadat@yahoo.com (Sadat Elutola Babalola)
s_ahmad@upm.edu.my (Muhammad Saiful Ahmad-Hamdani)
norazua@upm.edu.my (Norazua Zakaria)
*Corresponding author

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to new detections are crucial steps in curbing the spread of herbicide-resistant weeds. Advances in molecular biology and precision agriculture practices will significantly enhance efforts in managing herbicide-resistant weeds.

Keywords: *Elaeis guineensis*, herbicide resistance, integrated weed management, Malaysia, management strategies, oil palm plantations, oil palm sustainability

INTRODUCTION

Oil palm (Elaeis guineensis Jacq.) is a monocotyledonous perennial crop belonging to the Palm family, Arecaceae. Malaysia is the second largest palm oil producer, with a total planted area of 5.65 million hectares (Parveez et al., 2024). In 2023, the export volume was about 15 million tonnes, accounting for 29.5% of the global palm oil trade and contributing RM105 billion to the country's GDP (Parveez et al., 2024). These oils are used for cooking and various non-food purposes, such as animal feeds, cosmetics, detergents, and biodiesel (Murphy et al., 2021). Thus, the importance of this sector in driving the country's economic growth and development is undeniable.

However, palm oil production is threatened by herbicide resistance, which stems from excessive herbicide use in oil palm cultivation. It leads to reduced yields, increased management costs, biodiversity loss, and threats to food security and environmental sustainability (Dilipkumar et al., 2020; Seng et al., 2024; Tampubolon et al., 2024). Herbicide-resistant weeds are a significant challenge impacting management and productivity. They have been reported in major oil palm-producing countries like Indonesia, which shares similar agricultural practices with Malaysia (Casemiro et al., 2022). Indonesia has a lower overall incidence of resistance, with *Eleusine indica* (L.) Gaertn. (goosegrass) reported. However, resistance in *E. indica* is widespread, with numerous populations exhibiting resistance to both glyphosate and paraquat (Kurniadie et al., 2023; Umiyati et al., 2023). In contrast, Malaysia has documented resistance in four weed species, with three new cases emerging recently (Chuah et al., 2023; Heap, 2025; Seng et al., 2024). Therefore, this review evaluates the prevalence and impact of herbicide-resistant weeds in Malaysian oil palm plantations and explores integrated management strategies to mitigate this growing challenge.

Challenges in Oil Palm Cultivation

Given the increasing global demand for vegetable oils for food and industrial uses, it has become essential to maximise palm oil production. However, achieving this is a major challenge due to several constraints such as climate change, labour shortage, limited arable land, old age of crops, slow replanting process, and the occurrence of new and existing pests/diseases (Murphy et al., 2021; Parveez et al., 2024). Dilipkumar et al. (2020) identified weeds as a particular factor affecting productivity in oil palms.

Several studies have identified such weeds as *Ageratum conyzoides* L., *Asystasia gangetica* (L.) T. Anderson, *Chromolaena odorata* (L.) R.M. King and H. Rob., *Clidemia hirta* (L.) D. Don, *E. indica*, *Hedyotis verticillata* (L.) Lam, *Ischaemum timorense* L., *Melastoma malabathricum* L. and *Mikania micrantha* Kunth (Wibawa et al., 2010; Dilipkumar et al., 2020). If not controlled, these weeds compete with crops, reducing the quantity and quality of yields. They may also act as vectors of pests and pathogens that infest crops. Weeds also interfere with farming practices such as irrigation, fertiliser application, and harvesting (Rosli et al., 2010; Murphy et al., 2021).

Generally, weeds are controlled using various cultural, physical, biological, mechanical, and chemical strategies (Nobilly et al., 2022). However, physical and mechanical weed control methods are inefficient, labour-intensive, tedious, and weather-dependent, especially in commercial plantations, while biological and cultural methods are not particularly effective. Therefore, weed management in Malaysian oil palm heavily depends on herbicides because they are the most reliable and effective control method (Casemiro et al., 2022). Considering the significance of oil palm to Malaysia's economy, effective weed management is vital for the long-term productivity and sustainability of the oil palm plantations.

HERBICIDE RESISTANCE IN MALAYSIAN OIL PALM

Emergence of Herbicide Resistance

Malaysia is one of the top herbicide users in Southeast Asia due to its heavy dependence on herbicides involved in the cultivation of rice (*Oryza sativa* L.), rubber (*Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg.), and oil palm (Casemiro et al., 2022). In 2021, herbicides constituted 70% of the total pesticide use in Malaysia (Food and Agriculture Organisation of the United Nations [FAO], 2023). The quick success of herbicides and ease of operation make them an attractive option in weed control. However, misuse of these chemicals has led to the emergence of herbicide-resistant weeds in oil palm plantations.

Herbicide resistance in weed populations occurs when the weeds are repeatedly exposed to the same herbicide or herbicides with similar modes of action, which imposes a selection pressure. Resistance in Malaysian oil palm plantations is linked to heavy reliance on herbicides, inadequate knowledge regarding herbicide classes, and improper application techniques (Seng et al., 2024). For example, resistance to metsulfuron-methyl in the *C. hirta* population in Jerantut, Pahang, was observed after continuous use for at least five consecutive years (Ramadzan et al., 2012). Similarly, Purba and Sipayung (2022) reported that monthly applications of glyphosate for three successive years resulted in glyphosate resistance in *E. indica.* Furthermore, some growers often fail to follow recommended guidelines for herbicide application, increasing the risk of resistance development (Seng et al., 2024). The biology, ecology, lifecycle, and genetics of weeds also contribute to the resistance (Perotti et al., 2020).

In Malaysia, eight weed biotypes have developed herbicide resistance in oil palm nurseries and plantations (Ramadzan et al., 2012; Heap, 2025). The first case of herbicide resistance occurred in 2005 when *E. indica* showed multiple resistance to glyphosate and paraquat (Chuah et al., 2005). Since then, four weed species, *C. odorata*, *C. hirta*, *E. indica,* and *H. verticillata*, have evolved resistance to herbicides like glyphosate, paraquat, metsulfuron-methyl, glufosinate, and fluazifop (Table 1). Notably, all these weeds are considered noxious due to their aggressive growth, intense competition with crops, and resistance to control measures. A recent survey of oil palm plantations in Peninsular Malaysia reported the presence of five putative-resistant biotypes, such as *E. indica*, *C. hirta*, *M. malabathricum*, *Ischaemum muticum* L., and *A. gangetica* (Seng et al., 2024).

Globally, 534 unique cases of herbicide-resistant weeds involving 73 species have been reported in 100 crops in more than 70 countries (Heap, 2025). Presently, herbicide resistance is the principal factor affecting the successful control of weeds. Resistant biotypes threaten agricultural productivity because they can survive herbicide applications, reproduce, spread, and become dominant in weed communities, making weed management difficult. This consequently leads to lower crop yields and an increased cost of weed control (Kurniadie et al., 2023; Ofosu et al., 2023; Seng et al., 2024). Furthermore, the limited herbicide options in oil palm management and the lack of new modes of action make herbicide resistance a concern (Dilipkumar et al., 2020). Hence, implementing effective strategies that reduce the impact of herbicide-resistant weeds is crucial in preventing economic losses and sustaining productivity.

Although several reports have highlighted the negative impact of herbicide-resistant weeds on oil palm productivity (Dilipkumar et al., 2020; Umiyati et al., 2023; Seng et al., 2024), current data on their competitive effects on oil palm yield and quality are limited. Furthermore, there is no documented information regarding the resistance mechanisms of these weeds except for *E. indica*. Focusing on lesser-studied herbicide-resistant weed species is essential for effective crop management and sustaining agricultural productivity. Research should be conducted to quantify the impact of herbicide-resistant weed species on Malaysian oil palm plantations. Investigating the specific mechanisms is important in developing targeted management strategies against these weeds.

Mechanisms of Herbicide Resistance

The mechanisms involved in herbicide resistance are target-site resistance (TSR) and non-target-site resistance (NTSR) mechanisms. TSR in weeds is caused by mutations that alter the target site of the herbicide, thereby reducing its binding affinity for the herbicide. TSR may also be caused by overexpression, which requires a higher herbicide concentration to inhibit the enzyme. While, NTSR involves mechanisms such as reduced absorption and translocation, enhanced metabolism, and rapid detoxification of herbicides that prevent phytotoxic amounts of herbicide from reaching the target site (Murphy & Tranel, 2019). NTSR has been linked to cross-resistance in weeds. This occurs when resistance to one herbicide confers resistance to others with the same mode of action. Weeds can also evolve resistance to multiple herbicides with different modes of action caused by either a mutation or several TSR and NTSR mechanisms (Gaines et al., 2020). Cross-resistance and multiple resistance limit the efficacy of diverse herbicides, making weed control difficult.

In Malaysia, weeds are resistant to five herbicide classes with different modes of action. The following discussion briefly describes resistance to these classes.

Resistance to Groups of Herbicides

Acetyl-CoA Carboxylase (ACCase) Inhibitors

Acetyl-CoA carboxylase (ACCase) inhibitors are herbicides targeting the ACCase enzyme, essential for lipid biosynthesis. This disruption leads to cell membrane damage and, eventually, the death of the plants (Murphy & Tranel, 2019). There are three chemical families in ACCase inhibitors: (1) aryloxyphenoxypropionates (FOPs), (2) cyclohexanediones (DIMs), and (3) phenylpyrazolin (DEN). These herbicides are extensively used to control grasses due to their low soil toxicity and being selective in major crops.

However, resistance to these herbicides has been reported in at least 51 weeds worldwide (Heap, 2025) due to reduced ACCase sensitivity and enhanced metabolism (Perotti et al., 2020). Several studies have identified mutations at seven positions: (1) Ile-1781-Leu/Val, (2) Trp-1999-Ser, (3) Trp-2027-Cys, (4) Ile-2041-Asn/Val, (5) Asp-2078-Gly, (6) Cys-2088-Arg, and (7) Asn-2097-Asp (Cha et al., 2014; Araújo et al., 2023). ACCase-resistant populations are often resistant to multiple ACCase inhibitors, making their control difficult. Enhanced metabolism of herbicides is associated with cytochrome P450 monooxygenases and glutathione-S-transferases. In Malaysian oil palms, resistance to butroxydim, fluazifop-butyl, haloxyfop-methyl, and sethoxydim has been reported in *E. indica* populations (Cha et al., 2014; Jalaludin et al., 2015; Chuah et al., 2023)

Resistance to Acetohydroxyacid Synthase (AHAS) Herbicides

Acetohydroxyacid synthase (AHAS) or acetolactate synthase (ALS) herbicides inhibit the plant enzyme, thereby preventing the synthesis of the branched-chain amino acids, valine, leucine, and isoleucine, ultimately leading to plant death. They consist of six groups: (1) imidazolinones (IMIs), (2) pyrimidinyl benzoates (PTBs), (3) sulfonanilides, (4) sulfonylureas (SUs), (5) triazolinones (SCTs), and (6) triazolopyrimidines (TPs) (Riechers et al., 2024). These herbicides are effective, selective, broad-spectrum inhibitors of several grass and broadleaved weeds, ensuring their worldwide use (Riechers et al., 2024). However, resistance to AHAS herbicides may develop quickly after just a few applications.

The primary resistance mechanisms to AHAS are target site mutation and, to a lesser extent, enhanced herbicide metabolism by cytochrome P450s (CYP450) (Gaines et al., 2020). Target site mutations lead to amino acid substitutions that reduce the herbicide's ability to bind effectively to the AHAS gene. Twenty-nine amino acid substitutions have been identified at nine positions (Ala122, Pro197, Ala205, Phe206, Asp376, Arg377, Trp574, Ser653, and Gly654) in the AHAS genes across several weeds (Gaines et al., 2020; Zakaria et al., 2021; Heap, 2025). Enhanced metabolism by CYP450 enzymes has also been observed in resistant weed species.

Resistance to Glyphosate

Glyphosate is a systemic herbicide that targets 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme essential for the biosynthesis of aromatic amino acids needed for protein synthesis in plants. By inhibiting EPSPS, glyphosate prevents amino acid production, ultimately leading to plant death (Gaines et al., 2020). Glyphosates are used worldwide in several crops. In oil palm plantations, glyphosate has been used for several decades to control weeds due to its efficient control against several weeds (Purba & Sipayung, 2022; Kurniadie et al., 2023). However, the prolonged use resulted in resistance. Currently, glyphosate resistance is the second most reported, with approximately 60 weed species exhibiting resistance worldwide (Heap, 2025).

Various studies have shown that glyphosate resistance in *E. indica* is primarily caused by mutations in the EPSPS gene (Pro106, Pro381, Thr102, Ala103, and Gly 101) and an increased expression of the gene resulting in the overproduction of the EPSPS protein, thereby reducing glyphosate efficacy (Chen et al., 2024; Deng et al., 2022; Kurniadie et al., 2023). Mutations and EPSPS amplification can occur in the same individuals, leading to highly resistant weeds. Enhanced metabolism involving cytochrome P450s and glutathione S-transferases (GSTs), reduced translocation or uptake, or enhanced vacuolar sequestration is the NTSR mechanism (Deng et al., 2022; Chen et al., 2024; He et al., 2024).

Resistance to Glutamine Synthetase Herbicides

Glutamine synthetase herbicides, such as glufosinate ammonium, inhibit glutamine synthetase, a key enzyme catalysing glutamine synthesis from glutamate. This inhibition prevents the conversion of glutamate and ammonia, leading to the accumulation of ammonia and disruption of amino acid metabolism, ultimately leading to plant death, for example, glufosinate-ammonium. Ser59Gly mutation was identified as the resistant mechanism in

E. indica resistant populations from Malaysia and China (Zhang et al., 2022). Resistance may also be conferred by gene amplification/overexpression, leading to increased enzyme activity and reduced sensitivity to the herbicide. Herbicide metabolic resistance to glufosinate involves the activity of the GST genes (Lei et al., 2024).

Resistance to Photosystem I Electron Diverters

Photosystem I (PSI) electron diverters are herbicides that disrupt the photosynthetic process in plants, producing reactive oxygen species that cause the plant's death. This group includes herbicides such as paraquat and diquat (Nazish et al., 2022). These herbicides are nonselective and are used to control weeds in major crops. Resistance to paraquat may be due to the rapid sequestration of paraquat into vacuoles, decreased translocation, and enhanced scavenging of reactive oxygen species (Nazish et al., 2022). In Malaysia, *E. indica* and *H. verticillata,* are resistant to paraquat.

Table 1
Herbicide-resistant weed species in Malaysian oil palm plantations

Weed Species	Common name	Year reported	Mechanism of action	Active ingredient	Location
Hedyotis verticillata	Woody borreria	2005	PS I inhibitors EPSPS Inhibitors	Paraquat Glyphosate	Terengganu
Eleusine indica	Goosegrass	2009	ACCase Inhibitors	Butroxydim Fluazifop-butyl Haloxyfop-methyl	Pahang
			EPSPS Inhibitors	Glyphosate	
			PS I inhibitors	Paraquat	
			Glutamine Synthetase Inhibitors	Glufosinate-ammonium	
Clidemia hirta	Soapbush	2010	AHAS inhibitors	Metsulfuron-methyl	Pahang

AHAS: Aceto Lactate Synthase
ACCase: Acetyl-CoA carboxylase
EPSPS: Enolpyruvyl Shikimate Phosphate Synthase
PS I: Photosystem 1 Electron Diversion

DOCUMENTED CASES AND WEED SPECIES

Hedyotis verticillata (L.) Lam

Hedyotis verticillata (L.) Lam (synonyms; *Oldenlandia verticillata* L.; *Scleromitrion verticillatum* (L.) R. J. Wang), is commonly known as woody borreria. It is a dicot weed in the Rubiaceae family. This perennial weed is native to South and Southeast Asia (POWO, 2024). *H. verticillata* has a sprawling growth habit with stems ranging from 15 to 100 cm. The stem tends to be woody as it matures, making it quite challenging to manage. It is a noxious weed that infests oil palms.

Chuah et al. (2005) identified several herbicides for control, such as paraquat, glyphosate, metsulfuron-methyl, triclopyr, fluroxypyr, 1-methyl heptyl ester, and picloram. However, the use of paraquat has been stopped due to safety concerns for humans and the environment. These herbicides are used with cultural, physical, biological, and mechanical methods.

Hedyotis verticillata first evolved multiple resistance to PS I and EPSPS inhibitors in Malaysia in 2005. This resistance was first detected in the Federal Land Consolidation and Rehabilitation Authority (FELCRA) oil palm plantations in Terengganu, where these herbicides have been used repeatedly for at least three consecutive years. Chuah et al. (2005) attributed herbicide resistance in the population to the same herbicide use, tank mix, and herbicide rotation. *Hedyotis verticillata* showed multiple resistance to glyphosate and paraquat, likely involving both TSR (EPSPS gene mutations) and NTSR mechanisms. It may also be cross-resistant to other EPSPS inhibitors. However, specific studies are needed to confirm this. Limited information on its weed biology, genetics, and current resistance status complicates control efforts.

Using alternative herbicides and diversifying weed control methods could effectively manage the herbicide-resistant weed. Zakaria et al. (2020) demonstrated the efficacy of aqueous leaf extracts of *Murraya koenigii* (L.) Spreng in inhibiting the emergence and growth of *H. verticillata* seedlings. Furthermore, the compound 5-(3-Fluoro-phenyl)-7-methyl-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester ("c") showed high post-emergence and pre-emergence herbicidal activity against *O. verticillata* and *E. indica.* The herbicidal action of compound "c" is attributed to its ability to trigger electrolyte leakage in the plant cells, a novel mode of action compared to traditional herbicides (Abdullah et al., 2021). However, further research is needed to develop pre-emergence natural herbicides from these extracts that can help to mitigate resistance in this weed species.

Eleusine indica **L. (Gaertn)**

Introduction

Eleusine indica L. (Gaertn), commonly known as goosegrass, is an annual grass belonging to the Poaceae family. Although native to Africa, it is widespread in other tropical regions (Rojas-Sandoval & Acevedo-Rodríguez, 2014a). It is among the top 10 worst weeds prevalent in over 50 crops in at least 60 countries. *E. indica* has a prostrate or spreading growth, although it can also grow upright, reaching a height of up to 60 cm. The sheaths and stem bases are distinctly flattened. The plant has a tufted base from which culms arise, with each culm bearing an inflorescence. Its green, flat leaves can be up to 8 mm wide and 15 cm long (Rojas-Sandoval & Acevedo-Rodríguez, 2014a).

E. indica has a rapid, continuous life cycle, germinating and producing seeds throughout the year, with multiple generations possible in a single season. A mature plant can produce 50,000 to 140,000 seeds, which are easily spread by wind, water, and animals, creating a large seed bank in the soil, which readily germinate to produce abundant seedlings. Due to its fast growth, aggressiveness, and well-developed root system, it is almost impossible to eradicate once it is established. *E. indica* thrives in moist, fertile soil in full sunlight but can grow in various environmental conditions (Jalaludin et al., 2010). Its tolerance to many herbicides makes it a major concern for farmers.

Herbicide Resistance (Mechanisms and Studies)

In Malaysia, *E. indica*, locally known as "rumput cakar ayam" or "rumput sambau", is prevalent in nurseries of oil palm and rubber, orchards, and vegetable fields, causing a drastic effect on crop yields due to the lack of canopies (Jalaludin et al., 2010). *E. indica* infestation in farms and plantations is normally controlled through herbicide application. Unfortunately, the heavy herbicide use led to its resistance to several herbicides such as glyphosate, glufosinate, paraquat, and fluazifop (Jalaludin et al., 2015; Sim et al., 2020; Purba & Sipayung, 2022). The high variation in the molecular and morphology in *E. indica* may also contribute to this multiple resistance (Saidi et al., 2016).

Resistance to glufosinate ammonium in *E. indica* was first detected in an oil palm nursery in Jerantut, Pahang, in 2009. Further investigation confirmed the high and multiple resistance to paraquat and three ACCase inhibitors (fluazifop-P-butyl, haloxyfop-P-methyl, and butroxydim).

Resistance to glufosinate ammonium in *E. indica* has been studied less than in other herbicides (Zhang et al., 2022). However, enhanced metabolic pathways by GST and other NTSR mechanisms are important in glufosinate resistance in *E. indica* (Lei et al., 2024). Amino acid substitutions at Trp-2027-Cys and Asn-2097-Asp in the ACCase gene contributed to the resistance in *E. indica*, although NTSR mechanisms may also be involved (Cha et al., 2014; Jalaludin et al., 2015). Other mutations such as Trp-1999-Ser, Trp-2027-Cys, and Asp-2078-Gly have been reported in resistant *E. indica* from China, and Asp-2078-Gly in the resistant population from Brazil (Araújo et al., 2023; Deng et al., 2023). Also, some populations demonstrate enhanced metabolism, further complicating management.

Similarly, glyphosate resistance in *E. indica* is linked to mutations in the EPSPS gene and an increased expression of the EPSPS enzyme, reducing the weed's sensitivity to herbicides. A double mutation at Thr102Ille and Pro106Ser (TIPS) was identified in highly resistant plants (Jalalulidin et al., 2015; Zhang et al., 2021). Kurniadie et al. (2023) attributed

Thr102Iso and Pro106Ser amino acid mutations in the EPSPS gene as the mechanisms in glyphosate-resistant *E. indica* from oil palm in Indonesia. Enhanced metabolism involving the ATP-binding cassette transporter, aldo-keto reductase, and CYP genes are associated with NTSR (Deng et al., 2022; Chen et al., 2024; He et al., 2024).

Eleusine indica develops resistance through various TSR and NTSR mechanisms, leading to cross- and multiple resistance. Weeds resistant to glyphosate are often resistant to herbicides such as glufosinate and paraquat, ACCase inhibitors (Jalaludin et al., 2015; Zhang, et al., 2021; Kurnadie et al., 2023; Umiyati et al., 2023). They are also more likely to develop resistance to alternative herbicides, such as protoporphyrinogen oxidase (PPO) and PSII inhibitors, which makes their management challenging.

Management Strategies

Managing resistant *E. indica* involves using alternative herbicides and their mixtures. Herbicide mixture of MSMA+Diuron effectively controlled multiple resistant *E. indica* in young oil palms in Malaysia and Indonesia (Sim et al., 2020; Tampubolon et al., 2020). Several studies have reported the high efficacy of post-application herbicides such as ametryn, glufosinate ammonium, premixed MSMA + diuron, propaquizafop, sulfentrazone, and topramezone in controlling resistant populations (Purba & Sipayung, 2022; Kurniadie et al., 2023). Similarly, pre-application herbicides, including diuron, flumioxazin, indaziflam, oxyfluorfen, and pendimethalin, effectively suppressed glyphosate-resistant *E. indica* populations (Purba & Sipayung, 2022; Tampubolon et al., 2024). Furthermore, a combination of oil palm frond residues and reduced pre-emergence herbicide rates inhibited the emergence and growth of *E. indica* seedlings (Chuah & Lim, 2021).

The concerning levels and patterns of herbicide resistance observed in *E. indica* populations underscore the need to use different herbicides and adopt integrated weed management (IWM) methods, such as cover cropping and livestock grazing.

***Clidemia hirta* (L.) D. Don**

Clidemia hirta (L.) D. Don. (Synonym; *Miconia crenata* (Vahl) Michelang.), commonly known as “Koster’s curse” or soapbush, is a dicot weed belonging to the Melastomataceae family (Ramadzan et al., 2012). *C. hirta* originated from the Caribbean, Central and South America, but is now widely distributed in other tropical regions such as Australia, peninsular Malaysia, East and West Africa, and several oceanic islands.

Biology and Ecology

Clidemia hirta is a densely branched, hairy perennial shrub, between 1 to 5 metres tall. It is supported by shallow lateral roots and abundant fine roots with several stems arising from the rootstalk and the base of the main stem in mature plants. The leaves are about 5–18cm long and are oppositely arranged on slender twigs and branches. They are ovate, with a cordate base and elongated tip having either a serrated or entire margin and five distinct veins radiating from the base. The flowers are white or pink coloured and are grouped in small clusters at the tips of the branches (Rojas-Sandoval & Acevedo-Rodríguez, 2014b).

Clidemia hirta is considered one of the world's worst invasive species, reported in over 50 countries infesting several economic crops (Ramadzan et al., 2012; Rojas-Sandoval & Acevedo-Rodríguez, 2014b). Locally known as "senduduk bulu", *C. hirta* is one of the most noxious weeds competing with the crops for water, light, and nutrients in Malaysian orchards and plantations. Furthermore, it smothers and causes physical stunting of immature palms and reduces fruit yield in mature palms, leading to poor agricultural productivity (Ramadzan et al., 2012). In addition, *C. hirta* has been shown to exhibit antipathogen effects, which may enhance its competitive and invasive ability (Pratami et al., 2021).

The presence of *C. hirta* continues to be a major constraint in plantations due to its broad ecological tolerance, quick spread, prolific seed production (a single plant can produce between 100,000 to 500,000 seeds), lack of natural predators, and inclination to form dense and monotypic thickets (Conant, 2009). *C. hirta* is also shade-tolerant and thrives in mature plantations. These traits enable its survival and continue to spread when introduced into new cropping areas, making *C. hirta* a serious threat to oil palm productivity.

Herbicide Resistance in **C. hirta**

Clidemia hirta was first reported to have acquired resistance to metsulfuron-methyl in 2010, and subsequent experiments further established the resistance (Chuah & Sahid, 2010; Ramadzan et al., 2012). Resistance in *C. hirta* has been attributed to a target-site mutation that reduces the ability of the enzyme to bind effectively to the AHAS gene. Although amino acid substitutions have been reported at nine positions in the AHAS gene, resistance to the SU family is primarily due to changes at Pro197 and Trp574 (Tranel et al., 2025). Enhanced metabolism by the CYP450 enzymes may also be responsible for resistance. This biotype's moderate to high resistance levels indicated a potential cross-resistance to other herbicides in the same chemical family/class (Ramadzan et al., 2012). Given its potential for spread, herbicide-resistant *C. hirta* has likely spread to other areas but is under-recorded.

Effective control of this resistant weed has proven difficult. Mechanical methods are unsuccessful because it resprouts vigorously after being cut. Therefore, control of *C. hirta* mostly depends on fluroxypyr, metsulfuron, triclopyr, picloram, and 2,4-D (Department of Agriculture, Malaysia [DOA], 2025). Using alternative herbicides, rotating them, livestock grazing, and mulching will help prevent the weeds' growth, thereby delaying the onset and reducing the spread of resistant individuals.

Recently, the AHAS gene fragment of susceptible *C. hirta* and the nucleotide sequence (2384bp) comprising the nine potential mutation sites were registered in GenBank (Accession No. PQ153909). However, research on the specific mechanisms of herbicide resistance in *C. hirta* is still lacking. Thus, research elucidating its resistance mechanisms is crucial for weed management decisions.

IMPLICATIONS OF HERBICIDE RESISTANCE

Herbicide resistance in oil palm plantations significantly impacts agricultural productivity and environmental sustainability (Purba & Sipayung, 2022). With weeds becoming resistant, herbicide applications are becoming less effective. This forces managers to rely on alternative control methods, such as more expensive herbicides, frequent applications, and manual and mechanical hand-weeding, thereby increasing management costs (Purba & Sipayung, 2022; Kurniadie et al., 2023; Seng et al., 2024). Given that the recommended herbicides for oil palm plantations are few, resistance limits available options, thereby narrowing viable weed management strategies (Dilipkumar et al., 2020). Some resistant species, like *E. indica*, further resist multiple herbicide groups, limiting herbicide options (Jalaludin et al., 2015; Heap, 2025). The rise of herbicide-resistant weeds complicates integrated weed management (IWM) practices.

Also, herbicide resistance may lead to over-reliance on more toxic herbicides and environmental and health concerns such as food contamination, human health risks, and biodiversity loss (Zakaria et al., 2020; Umar et al., 2023). For instance, the evaluation of oil palm plantation soils in Sepang, Selangor, showed the presence of diuron for up to 90 days after application (Muhamad et al., 2013). Some herbicides, including glyphosate, glufosinate ammonium, fluroxypyr, metsulfuron-methyl, and triclopyr, have also been detected in water bodies, soils, and sediments linked to oil palm plantations (Mardiana-Jansar & Ismail, 2014; Mardiana-Jansar & Ismail, 2016; Tayeb et al., 2017; Dearlove et al., 2024).

Furthermore, herbicides may also destroy non-target understory vegetation vital for supporting biodiversity within oil palm ecosystems (Umar et al., 2023). Long-term use of herbicides harms beneficial soil microorganisms, inhibiting their growth and disrupting the natural balance between pathogens and beneficial organisms (Zain et al., 2013). This consequently impacts microbial activity and soil health. Furthermore, herbicides can make oil palm seedlings more susceptible to diseases like *Ganoderma boninense* by causing wounds that allow pathogen entry, thus compromising crop health (Hussin et al., 2021).

As herbicide-resistant weeds continue to spread, sustainability efforts are undermined. Increased chemical use and harmful practices can prevent plantations from meeting sustainability standards, like the Roundtable on Sustainable Palm Oil (RSPO) and Malaysian Sustainable Palm Oil (MSPO) certifications, which aim to reduce herbicide use (Azhar et al., 2021; Nobilly et al., 2022). The spread of herbicide resistance also poses a significant risk to neighbouring or newly established plantations. Resistant weed populations may contaminate these areas through seed dispersal by wind, water, or human activities. This cross-contamination exacerbates the resistance issue and introduces potential environmental and crop health risks, making management efforts more challenging.

Lastly, herbicide resistance is expected to influence agricultural policies as Malaysia strives towards sustainable agriculture, food security, and environmental conservation. Future strategies are likely to focus on more integrated and sustainable weed management practices such as biological control, molecular biology, and precision agriculture (Perotti et al., 2020; Ofosu et al., 2023). These approaches aim to reduce reliance on chemical herbicides, protect biodiversity, and address the issue of herbicide resistance. Effective management of herbicide resistance requires a strong technological and regulatory framework. This includes stricter regulations on herbicide use, monitoring systems to track resistance trends, and enforcing compliance with sustainable farming practices. Increased government funding could support research in alternative weed control methods, as well as provide subsidies for adopting these practices. Additionally, regulations promoting the use of precision agriculture, such as drones and sensors, can improve herbicide application efficiency, ultimately mitigating resistance development and spread.

MANAGEMENT OF HERBICIDE-RESISTANT WEEDS IN OIL PALM

Weed management is a crucial component of oil palm cultivation, and various approaches are involved. The concerns posed by herbicide resistance have made implementing diverse and effective weed management strategies necessary to ensure the sustainability of oil palm cultivation in Malaysia. Current weed management in oil palm plantations comprises several methods described below.

Mechanical Control

Mechanical methods like chaining, bulldozing, and ploughing control weeds in plantation crops. Manual weeding, such as slashing of woody weeds, hoeing, and hand pulling, reduces the weed cover and prevents fruiting, thereby slowing the growth and spread of the weed. Plant litter decomposition also releases nutrients into the soil, increasing soil fertility. Mechanical weeding is important in clearing established woody plants such as *C. hirta* and *M. malabathricum*. A major advantage of mechanical control is its ability to reduce herbicide use, thereby lowering the risk of herbicide resistance. By minimising herbicide use, mechanical weeding also reduces environmental impact, allowing beneficial organisms to thrive, preserving soil health, and protecting water resources, ultimately contributing to a more balanced and resilient ecosystem (Formaglio et al., 2020; Iddris et al., 2023).

While mechanical methods offer long-term sustainability by reducing reliance on chemicals and improving biodiversity, they are inefficient, labour-intensive, tedious, and weather-dependent, especially in commercial plantations (Dilipkumar et al., 2020; Ruzlan & Ahmad-Hamdani, 2021). Furthermore, the farm labour shortage affects its practicality (Murphy et al., 2021; Parveez et al., 2024). Mechanical weeding does not remove the roots, allowing plants to grow back quickly. Thus, frequent cutting is essential to maintain effective weed control.

Cultural Control

Cultural weed control in oil palm plantations is a long-term strategy that focuses on preventing weed establishment. Leguminous cover crops, such as *Centrosema pubescens* Benth., *Calopogonium mucunoides* Desv., *Mucuna bracteata* DC. Ex Kurz*., Pueraria phaseoloides* (Roxb.) Benth. and *Stylothanthes* reduce weed growth and cover in immature oil palms (Ruzlan & Ahmad-Hamdani, 2021; Nobilly et al., 2022). Cover crops prevent the sprouting and spread of noxious weeds. It also reduces soil erosion and fixes nitrogen, thereby increasing soil fertility. In addition, the remains of cover crops in the soil increase organic matter and may control weeds by releasing allelochemicals, making plantations more resilient against weed infestations. Considering evolving herbicide resistance patterns, this method minimises the need for herbicides, reducing the risk of resistance development.

Several studies reported that different cover crop systems lead to variations in weed communities and oil palm yields, with *Axonopus compressus* (Sw.) P. Beauv. and *M. bracteata*, showing potential for weed suppression (Samedani et al., 2014; Samedani et al., 2015). However, cover crops require regular de-creeping, either manually or by herbicides, to prevent them from smothering plantation crops due to their creeping/climbing behaviour (Dilipkumar et al., 2020). Mulching is another cultural practice in which cut fronds and empty fruit bunches suppress weed growth, recycle nutrients, and prevent leaching (Ruzlan & Ahmad-Hamdani, 2021).

While cover crops and mulching help suppress weed growth, they may not be sufficient to manage all types of weeds, particularly noxious herbicide-resistant ones. Moreover, cultural controls often require significant changes in plantation practices, which can be challenging to implement on a large scale. Investigating the combination of cover crops with other weed management strategies will provide valuable insights into controlling and preventing herbicide resistance.

Biological Control

The integration of livestock such as sheep, goats, and cattle has been reported in weed control in oil palm plantations. Livestock grazing offers economic, environmental, and ecological benefits while meeting sustainability goals. Livestock grazing in palm oil production helps meet sustainability standards such as RSPO by reducing herbicide use (Álvarez et al., 2024). It also reduces labour and weeding costs in oil palm plantations. Álvarez et al. (2024) reported a 60% reduction in weed control costs and a 15% income increase due to cattle integration. Furthermore, reduced herbicide use lowers workers' health risks and minimises environmental contamination (Tohiran et al., 2017). It also maintains the understory vegetation, protecting the oil palm ecosystem's biodiversity (Nobilly et al., 2022; Umar et al., 2023), and animal droppings contribute to soil fertility (Tohiran et al., 2017).

Recently, a targeted goat grazing study conducted in a mature oil palm plantation in Pahang, Malaysia, observed that noxious weeds such as *A. gangetica* and *C. hirta* were effectively cleared (Nobilly et al., 2022). Likewise, Tohiran et al. (2023) reported that multi-species livestock grazing (sheep and goats) effectively controlled the understorey plants in oil palm plantations compared to grazing by a single species. However, livestock grazing in oil palm largely depends on the animal species and density, grazing plans, and duration. Therefore, integration techniques and the stocking density of animals should be evaluated. Grazing alone often fails to provide adequate long-term control; therefore, combining it with other weed management strategies is important in weed control. Further research and proper management practices are essential for broader implementation.

Chemical Control

This is the most widely used method to control weeds in oil palm plantations (Dilipkumar et al., 2020). Once herbicide resistance is detected, the use of that herbicide should be discontinued. Chemical control includes rotating herbicides with different modes of action and tank mixtures (Kurniadie et al., 2023; Tampubolon et al., 2024). This reduces and delays resistance by lessening the selection pressure caused by herbicides with a single mode of action. The plantations must be monitored regularly after herbicide treatments to eradicate seedlings and probable herbicide-resistant weeds using other methods to prevent the spread. Furthermore, choosing herbicides for mixing requires careful consideration, as some combinations can exhibit antagonistic effects (Dilipkumar et al., 2020). Given the limited available herbicide options, research evaluating herbicides and tank mixtures with different sites of action offering a broad spectrum of weed control is important. It is also crucial to prioritise the development of novel herbicides to improve chemical control.

Integrated Weed Management

With herbicide resistance spreading, conventional weed control becomes less effective. Management practices integrating different control measures, such as chemical, cultural, mechanical, and biological methods, are a practical option for controlling resistant weeds in oil palm cultivation. This strategy reduces herbicide selection pressure by minimising herbicide use. The most widely practised IWM method by plantation companies combines herbicide use and cultural practices with or without biological control. A survey of some selected FGV estates in Lepar Utara, Pahang, indicated that IWM methods effectively managed weeds while reducing weeding costs by RM34.00/ha/year (Ruzlan & Ahmad-Hamdani, 2021). This approach also helped to reduce the occurrence of herbicide-resistant weed species, making it a viable long-term strategy. Chuah and Lim (2021) reported that oil palm frond residues could reduce pre-emergence herbicide rates required to inhibit *E. indica* seedling emergence and growth. Herbicide-treated mulch is a viable option for sustainable control. IWM strategies should also include monitoring for early detection of herbicide resistance. Given the heavy reliance on herbicides, there is a strong need for policies that would promote integrated weed management practices.

Bioherbicides

Bioherbicides involve the application of allelochemicals and natural products from plants, microbes, and insects for weed control (Ofosu et al., 2023). They are economical and eco-friendly, reducing herbicide use and environmental pollution (Ismail et al., 2020; Rusli et al., 2022; Ofosu et al., 2023). As herbicide resistance continues to evolve, bioherbicides can be formulated to target specific species without increasing the dependence on chemicals, offering a promising long-term solution for weed control in oil palm plantations.

Several studies have explored the potential of microorganisms to control weeds. Rusli et al. (2022) reported that PH81, a formulation of the fungus *Phoma herbarum*, showed 91.7% and 80% mortality in nursery and field trials against glyphosate-resistant E. indica. PH81 was also compatible with the herbicide diuron. Two fungal pathogens, *Bipolaris bicolour* and *Bipolaris sorokiniana,* have been recommended for their high efficacy against *E. indica* (Ismail et al., 2020; Fakri, 2022). Fakri (2022) reported that combining *B. bicolor* with ametryn enhanced its effectiveness, resulting in a 50%–94% reduction of leaf colour, shoot height, and weight. Furthermore, Norhafizah et al. (2020) investigated the activity of allelochemical 2,4-di-tert-butylphenol (2,4-DTBP) on the emergence of *E. indica*, and *O. verticilla*ta. Results indicated its efficacy at a rate of 2.5 kg ai/ha, comparable to commercial herbicides. Although bioherbicides are crucial in sustainable weed management, their implementation is low due to environmental factors, formulation challenges, toxicity concerns, and cost. Thus, requiring further research.

Emerging Technologies

Emerging technologies are changing agriculture by addressing traditional challenges such as herbicide resistance, environmental contamination, and high operational costs. These include precision agriculture for effective weed control and molecular markers in herbicide resistance detection.

Precision Agriculture

Emerging technologies in precision agriculture, such as global positioning systems (GPS), Geographic Information systems (GIS), sensor technologies, automated weed control systems, drones, satellites, Internet of Things, and robots, have various applications in oil palm plantations. They are useful in real-time monitoring of soil, meteorological conditions, and crops, allowing for efficient use of farm inputs and reducing waste. Using Unmanned Aerial Vehicles (UAVs), such as sensing drones for high-resolution imaging, is crucial for accurately detecting and mapping weed infestations. These high-resolution images help in creating precise weed cover maps. Based on the weed maps, actuation drones spray herbicides only when necessary, significantly minimising use. Ruzlan et al. (2024) reported that the use of UAVs (drones) for weed control in immature oil palm plantations reduced work time by 37%, water use by 91%, and costs of labour by 81% compared to traditional methods.

Furthermore, the adoption of artificial intelligence is crucial in tackling herbicide resistance. High-quality annotated datasets are generated for image recognition and machine learning. These datasets are crucial for training AI models to correctly detect and identify weeds. AI models can predict weed outbreaks and recommend optimal control measures, enhancing crop yields and sustainability. Firmansyah et al. (2022) proposed a machine learning-based system for weed identification and recognition, which could be deployed in real-time on mobile devices. Jopony et al. (2023) also proposed an automated system to classify immature oil palms based on datasets comprising images of young oil palms with noticeable palm circles.

Incorporating these novel technologies with conventional weed management will result in early detection of herbicide resistance and more effective control of herbicide-resistant weed biotypes in Malaysian oil palm plantations. These technologies are promising options for large-scale weed control due to their efficiency, effectiveness, and economic benefits while reducing environmental damage and promoting sustainable agriculture. However, challenges facing the implementation of UAVs and artificial intelligence in weed control are the significant initial costs and the technical expertise needed to set them up. Therefore, further research is important in developing affordable and easily adaptable technologies.

Molecular Biology

Also, omics technologies such as genomics, proteomics, metabolomics, and transcriptomics are important in understanding and identifying molecular mechanisms that endow herbicide resistance in weeds. RNA sequencing gives great insight into the molecular basis of

NTSR, particularly in weeds with limited or no prior genetic information (Gaines et al., 2020). Current research in next-generation sequencing and transcriptomics is important in elucidating the molecular and genetic regulations of NTSR.

Most often, herbicide resistance is only detected after weeds survive applications that previously controlled them. Early detection using molecular markers (DNA, RNA, and protein markers) that can identify resistance genes and allow for quick assessment of multiple resistances is crucial to preventing its spread (Lowe et al., 2024; Milani et al., 2024; Simard et al., 2024). These methods are faster and more cost-effective than traditional lab-based approaches, ensuring targeted strategies for each weed species, thus reducing ineffective herbicides and resistance. Protein biomarkers have been identified for detecting enhanced metabolic resistance to certain herbicides (Lowe et al., 2024). Enzymes, such as CYP450s, GSTs, and ABC transporters, can also be used as molecular markers in NTSR. The incorporation of omics is crucial for a better understanding of weeds and for improving weed management strategies in the future.

CONCLUSION

The heavy reliance on herbicides for weed control in oil palm plantations has led to the emergence of herbicide resistance in Malaysia. The incidence of herbicide-resistant weeds is increasing due to the insufficient implementation of diverse weed control practices. Inaction in addressing this issue in oil palm plantations may result in serious consequences such as decreased fruit yields, higher operational costs, challenges in effective integrated weed management, environmental contamination, and loss of biodiversity.

Adopting a comprehensive, long-term strategy that addresses current issues and protects the environment while ensuring sustained crop productivity to tackle herbicide resistance is essential. This should integrate sustainable practices, proactive research, innovative technologies, and policies that anticipate future threats to agriculture. Plantation managers should adopt sustainable practices, such as integrated weed management and efficient use of farm inputs, to maintain the overall health and productivity of oil palm plantations. This proactive approach allows timely adjustments to weed management strategies, ensuring their effectiveness. Integrating various practices such as cover cropping, mulching, livestock grazing, the use of alternative herbicides and their mixtures, and the yearly rotation of herbicides helps to reduce the selection pressure on weeds. Weed monitoring to eradicate noxious weeds before they fruit should be conducted regularly to prevent the establishment of such weeds. Training programs on proper herbicide use and integrated weed management can address limited knowledge about herbicide classification and resistance management. Herbicide stewardship should be followed strictly.

Prioritising research and innovation will reduce the onset and spread of herbicide resistance. An extensive understanding of the biology of weed species would provide valuable insights for developing effective controls to reduce the spread of herbicide-resistant weeds. Studies quantifying the competitive effects of lesser-studied weeds and confirming putative resistance are important. Furthermore, there is a pressing need for continued research into herbicide resistance mechanisms as this will help to develop targeted control measures. Future studies focusing on developing rapid diagnostic tests using molecular markers for resistance detection and artificial intelligence (AI) in weed control are crucial in mitigating herbicide resistance.

Science-based policy recommendations, such as those endorsed by the RSPO and MSPO, are important in tackling resistance by minimising herbicide use. Regulations regarding sustainable practices should be monitored to ensure compliance. Equally important is the collaboration of all stakeholders in integrating various management strategies.

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Combining Ability Estimates for *Striga* Resistance in Maize (*Zea mays* L.): A Full Diallel Analysis

Muhammed Itopa Jatto[1], Abdul Shukor Juraimi[2]*, Mohammed Y. Rafii[1], Mohamed Hazim Nazli[2], Yusuf Oladosu[1], Fakuta Naiwa Markus[3], Samuel C. Chukwu[4], Debi Rani Datta[1] and Mst. Motmainna[1,2]

[1]*Laboratory of Climate-Smart Food Production, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, Serdang 43400, Malaysia*
[2]*Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, Serdang 43400, Malaysia*
[3]*Rubber Research Institute of Nigeria, Gum Arabic Sub-Station, P.O. Box 244, Gashua, Yobe State, Nigeria*
[4]*Department of Crop Production and Landscape Management, Faculty of Agriculture and Natural Resources Management, Ebonyi State University, Abakaliki P.M.B. 053, Nigeria*

ABSTRACT

The development of hybrid maize cultivars resistance to *Striga* is a promising solution to reducing annual crop loss in endemic areas by 20-100%. This study aims to develop high-yielding *Striga* resistance hybrid maize cultivars to reduce annual crop loss due to *Striga* in endemic areas. Seven maize parents, including three inbred lines; TZSTR 190, TZSTR 193, and TZEI 114, and four open-pollinated varieties; SAMMAZ 14, SAMMAZ 16, SAMMAZ 17, and SUWAN, were identified and crossed in a 7×7 full-dial cross according to Griffin Method 1, Model I. The 7 parents, their F_1's, reciprocals, and 3 checks; GWG 111, GWG 888, and 5005 hybrids were evaluated in a glasshouse trial for *Striga* infestation reaction screening in 2019 and 2020. Significant variations were observed across environments, treatments, and genotypes for most characters, suggesting highly variable genotypes suitable for *Striga* resistance selection due to their varying responses in different environments. Cross combinations of high-yielding but *Striga*-susceptible parents like SAMMAZ 14 and SUWAN showed some resistance to *Striga*. The study identified several resistant hybrids with low *Striga* count and damage, as well as significant performers in grain yield per hectare and most yield components. The study found significant variance in GCA (General Combining Ability), SCA (Specific Combining Ability), and

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E-mail addresses:
jmitopa@fceyola.edu.ng (Muhammed Itopa Jatto)
ashukur@upm.edu.my (Abdul Shukor Juraimi)
mrafii@upm.edu.my (Mohammed Yusop Rafii)
m_hazim@upm.edu.my (Mohamed Hazim Nazli)
yoladosu@uiowa.edu (Yusuf Oladosun)
fakuta.markus@rrin.gov.ng (Fakuta Naiwa Markus)
schukwu@unam.na (Samuel Chibuike Chukwu)
debidatta.bari@gmail.com (Debi Rani Datta)
motmainna@upm.edu.my (Mst. Motmainna)
*Corresponding author

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reciprocal effects variance in *Striga* damage and *Striga* count in inoculated plants, indicating the importance of additive and non-additive gene actions and maternal gene effects.

Keywords: Additive gene, combining ability, nonadditive gene, open-pollinated variety, yield components

INTRODUCTION

Striga hermonthica (Del) Benth is a harmful parasitic weed. It severely damages staple cereal crops like maize and sorghum, reducing their yield. This species belongs to the Orobanchaceae family. It is prevalent in tropical and subtropical regions of West and Central Africa, India, Myanmar, Indonesia, and the United States (Gowda et al. 2021).

Striga, a dust-like seed plant, attacks host crops after seed germination, stealing water, nutrients, and carbohydrate requirements (Mudereri et al. 2020). Its phytotoxic effect causes significant yield losses (Sangaré et al. 2018). *Striga* seeds can remain dormant in the soil for 15-20 years. Its germination is triggered by the Strigolactones exudate from host plant roots (Yoneyama et al. 2016).

Developing hybrid host cultivars resistant to *Striga* could significantly reduce crop loss due to this pest. Information on GCA (General Combining Ability) and SCA (Specific Combining Ability) is vital in selecting parents or hybrids for effective breeding programs. Combining ability analysis is a veritable tool employed by breeders to identify superior parents that are better combiners used in hybridization programs. This allows for the exploitation of heterosis and selects better-performed crosses for direct use for commercialization or further breeding work.

Diallel mating design is a statistical method employed by most plant breeders. It statistically separates the performance of parents and progenies into GCA and SCA components. (Murtadha et al. 2018). This is one of the widely used mating designs for the study of genetic architecture in maize. It offers a more effective method of developing high-yielding hybrid(s) in maize. Breeders achieve this by crossing all possible combinations amongst the parental lines from different heterotic groups (Olayiwola et al. 2021).

There are several diallel mating designs. However, full diallel offers a more comprehensive insight into the genetics of a plant. It considers the reciprocal, maternal, and nonmaternal effects. In this study, a full diallel mating design was used in crosses between *Striga*-resistant inbred lines and high-yielding but susceptible ones. This helped to determine the gene action governing the inheritance of yield and *Striga* resistance. It also identified the best parent combinations for these characters.

Crop protectionists, weed scientists, and agronomists have all tried various approaches to eradicate this unwanted weed. However, most efforts have been unsuccessful. Even when positive results are obtained, they have no economic benefits to the poor farmers in the rural areas. These methods significantly increase the production cost (Mrema et al.

2020). Therefore, breeding for *Striga* resistant maize hybrids offers a more sustainable solution to mitigating crop losses, improving food security, and enhancing the livelihoods of farmers in *Striga* infested regions. The specific objective of this study was to investigate the effects of combining ability as well as estimate the genetic parameters (system) and mode of inheritance governing Striga resistance, earliness, growth, yield, and yield-related components in maize to identify superior parental lines (best general combining parents) and hybrids (best specific combining parents) for developing high yielding Striga resistant maize varieties.

MATERIALS AND METHODS

Seven parental lines of maize, as described in Table 1, were collected based on their yield performances and reaction to *S. hermonthica.* The parental lines were crossed in all possible combinations using a full diallel mating design as described by Griffin 1958 to produce 42 hybrids (21 cross and 21 reciprocals). This research was carried out at the Faculty of Agriculture, Universiti Putra Malaysia (UPM) research farm. The 42 hybrids (21 cross and 21 reciprocals), their parents, and 3 check varieties were screened in a glasshouse for *Striga* resistance in a polyethylene *Striga* seeds inoculated potted trial at the Faculty of Agriculture, Universiti Putra Malaysia (UPM). This trial was done for two planting cycles (2019 and 2020).

Plastic pots measuring 16 cm by 16 cm were filled with about 10 kg of top soil which was mixed with lime and poultry dung. A total of 312 polyethene pots were used. 156 pots were inoculated with striga seeds and arranged in a Completely Randomized Design and replicated three times.

For uniform dispersion, a mixture of fine dry river sands and 100-150 sterilized viable Striga seeds in a ratio of 1:99 was worked down 6 to 12 cm deep in the pots. The inoculated

Table 1
Characteristics of parent maize varieties used in the study

Ent no.	Name	Maturity	Karnel colour	Reaction to *Striga*	Source
1	TZSTR 190	Late	White	Resistant	IITA
2	TZSTR 193	Late	White	Resistant	IITA
3	TZE114	Late	White	Susceptible	IITA
4	SAMMAZ 14	Medium	White	Resistant	IAR
5	SAMMAZ 16	Medium	White	Tolerant	IAR
6	SAMMAZ 17	Late	White	Susceptible	IAR
7	SUWAN	Late	Yellow	Susceptible	THAILAND
8	GWG111	?	Yellow	?	COMMERCIAL
9	5005	?	Yellow	?	COMMERCIAL
10	GWG888	?	Yellow	?	COMMERCIAL

pots were left to settle for 14 days before the maize seeds were planted. Two seeds from each of the 52 genotypes (42 crosses, 7 parents, and 3 checks) were sown in *Striga*-inoculated potted soil at a depth of 4 cm. One week after planting, the number of plants per pot was thinned to one plant per pot. Drip irrigation was used to control the watering as needed. Weeding was done frequently to protect the potted plant from weed competition. Fertilizer applications of NPK 15:15:15 were made at rates of 40N, 40P, and 40K at two and four weeks following planting, respectively.

Data Collection

Striga emergences were counted in each plot at 7 and 10 WAP (weeks after planting). The damage rate was assessed visually during each trial on the two central rows at 7 and 10 WAP, on a scale from 1 to 9 (Kim, 1994). Days to tasseling, days to silking, days to maturity, number of ears, plant height (m), ear height (m), cob length (cm), cob weight per plant (g), 100-grain weight (g), shelling percentage (%), harvest index and grain yield per hectare (g) were recorded.

Statistical Analysis

A combined analysis of variance (ANOVA) was conducted on all data to evaluate the variability among parents and their offspring utilizing SAS (Statistical Analysis Software) version 9.4 (SAS Institute Inc., Cary, NC, USA). Means comparison was performed with Fisher's Least Significant Difference (LSD) at a 5% significance level. The general combining ability of parents and the specialised combining ability of hybrids were assessed using Griffing's method 1 model 1 (fixed effects), as outlined by Singh and Chaudhary (1977), employing R software.

RESULTS AND DISCUSSION

Analysis of Variance

The mean squares across the environment for most of the characters in this study, as shown in Table 2, were highly significant except for days to 50% tasselling, shelling percentage, and harvest index. This indicates that there are variations in the environments, and the genotypes perform differently in different environments all characters except for the above-listed characters. This is in agreement with the findings of Menkir and Meseka (2019) who reported that most traits in an infested environment were significantly affected by the environment. The mean squares for treatment, as shown in Table 2, were also highly significant for all the characters. This indicates that there is great variation in the performance of the genotypes in the different treatments. Similar results were reported by Adetimirin et al. (2000); Badu-Apraku et al. (2011); Menkir and Meseka (2019).

Table 2
Mean squares of analysis of variance for Striga *resistance, growth, yield, and characters of full diallel cross involving seven parents of maize and three checks varieties in combined analysis*

SOV	df	SCT7	SCT10	SDM7	SDM10	DTFT	DTFS	DTFM	PLHT	EHT	CLHT	CWPP	GYPH	HGWT	SHGP	HIDX
Season	1	42.94**	91.91**	44.69**	24.64**	9.75	3414.17**	166.94**	42948.03**	26386.62**	314.23**	29428.89**	71.31**	3381.30**	44.18	0.008
Rep.	2	4.10	21.18**	0.84	2.51	69.14**	230.79**	10.68	1189.85	47.77	1.04	2736.00**	26.19**	96.60	750.53	0.022*
Rep (Sea)	2	6.77*	13.63**	0.44	2.28	46.19**	9.70	2.72	75.66	143.31	22.10*	4570.46**	4.87**	6.46	251.00	0.010
Gen	51	3.46*	3.86	1.87**	5.94**	6.13**	10.86**	41.08**	2366.64**	841.25**	38.00**	1823.16**	4.15**	109.85**	754.22**	0.016**
Gen × sea	51	2.62	4.97**	0.89	2.20*	3.26	5.51	40.95**	1494.78**	412.51**	11.49**	648.62**	1.75**	45.56	484.56	0.006
Error	204	2.25	2.96	1.03	1.45	2.93	3.65	12.00	873.83	333.55	9.95	317.52	0.77	29.49	373.22	0.005
Total	311															

SCT7= *Striga* count at 7weeks, SCT10= *Striga* count at 10weeks, SDM7=*Striga* damage at 7 weeks, SDM10=*Striga* damage at 10 weeks, DTFT=Days to 50% tasselling, DTFS=Days to 50% Silking, DTFM=Days to 50% maturity, PLHT=Plant height, EHT=Ear height, CLHT=Cob length, CWPP= Cob weight/plant, GYPH=Grain yield/ha, HGWT=100 grain weight, SHGP=Shelling%, HIDX=Harvest index

The mean square for genotype for most characters was highly significant, except *Striga* count at 10 weeks after planting, suggesting that there was great variation among the genotypes, hence the difference in their performances. Similar trends were reported by Badu-Apraku et al. (2015); Menkir and Meseka (2019); Solomon et al. (2020).

However, the point of divergence with the present study was in the non-significance of the above listed *Striga* characters. This could be attributed to the death of some of the parasites before week 10 and before the host plant maturity, when the *Striga* plants were harvested. The death of the parasites could probably be due to high humidity in the greenhouse. Similarly, the mean square for genotype × environment was highly significant except *Striga* count at 7weeks, *Striga* damage at 7weeks, days to 50% tasselling, and days to 50% silking which indicate the dependence of the performance of the genotypes on the environment, this highlight the need for multi-environment trials to ensure stable performance across different agro-ecological zones. (Menkir and Meseka 2019; Ngugi et al. 2013, 2015).

Genetic Component

The mean square due to general combining ability (GCA) variance (additive gene effects) were not significant for all characters under study except for *Striga* damage at 10 weeks and days to 50% tasselling, which were significant at p=0.01 (Table 3). However, the variance due to specific combining ability (non-additive gene effects) for most characters were significant at p=0.01 and p=0.05 (days to 50% tasselling) except Striga count at 7, cob length, plant height, and ear height. The predominance of non-additive gene effect over additive gene effect in most of the characters under study except in Striga count at week 7, Striga damage at week 10, days to 50% tasseling, plant height at maturity, ear height at maturity and cob length where GCA was greater than SCA which suggests the importance of additive gene action in these characters (Olaoye and Bello 2009). SCA for Striga count at 10 weeks was higher when compared to the GCA, suggesting the preponderance of non-additive genes in controlling the numbers of Striga emergence. This is in agreement with the findings of Badu-Apraku et al. (2011). The ratio of δ2 GCA/ δ2SCA for all the characters were less than unity, which indicates the preponderance of non-additive gene effects in all the characters. Grain yield per hectare showed zero (0) GCA/SCA variance ratio, which suggests the involvement of both additive and non-additive gene action (codominance) in the two characters. The predominance of non-additive gene effects for most traits suggests that hybrid breeding strategies, such as reciprocal recurrent selection and heterosis exploitation, could be effective in improving maize performance (Fasahat et al. 2016).

The reciprocal mean square of most characters was highly significant (p=0.01). However, there was no significant difference in Striga damage at 7 weeks, days to 50%

Table 3
Mean squares value of analysis of variance, general combining ability, specific combining ability, reciprocal, maternal, and interactions for Striga *resistance, earliness, growth, yield and yield component characters for inoculated and non-inoculated potted experiment*

Variation	df	SCT7	SCT10	SDM7	SDM10	DTFT	DTFS	DTFM	PLHT	EHT	CLHT	CWPP	GYPH	HGWT	SHGP	HIDX
Env	1	10.80**	8.64**	3.32**	11517.60*	0.11*	9.17	8.66	173.18	10475.47*	14984.26**	1444159.51**	2.31E+09**	12306.26*	74696219862*	3393977.16
Rep (Env)	4	0.94	0.07	0.24	6123.54	0.13**	3.64	2.17	5003.95	830.64	422.53	8844.99*	2.58E+08	3714.37	24554981204	77498809.86*
Gen	48	0.59	0.22*	0.51**	4728.80	0.03**	7.49**	15.28**	2855.81	1959.92	589.10	10095.16	6.84E+08*	5197.79*	23239543026**	55259756.33**
GCA	6	0.82	0.17	1.64**	4233.44	0.07**	4.07	5.13	3239.15	2454.70	560.32	6585.63	2.47E+08	468.62	6621314303	17491675.72
SCA	21	0.45	0.27**	0.38**	4381.44**	0.03*	9.53**	17.48**	3037.93	1495.68	541.26	12128.82**	9.35E+08**	6669.27**	37611075599**	75562447.88**
REC	21	0.67*	0.18	0.32**	5217.70*	0.01	6.43**	15.97**	2564.17	2282.80	645.16	9064.23**	5.58E+08**	5077.51**	13616075803	45747944.95*
Mater	6	0.94*	0.31*	0.45**	3849.07	0.02	8.65**	17.97**	3104.98	2334.09	920.83	6815.40	6.68E+08*	2176.69	13298920263	54895113.93
No Mat	15	0.57	0.13	0.28*	5765.15*	0.01	5.54*	15.17**	2347.85	2262.28	534.90	9963.76**	5.14E+08*	6237.84**	13742938019	42089077.35
Gen × Env	48	0.41	0.14	0.25**	3782.78	0.01	4.04	6.42	2950.25	2448.15	435.50	7922.08**	4.14E+08*	3168.77	12222551047	28376293.55
GCA × Env	6	0.19	0.10	0.33*	2656.98	0.02	1.50	5.59	2533.58	786.96	435.69	2934.91	2.95E+08	1966.39	9955467725	21933350.34
SCA × Env	21	0.60	0.21*	0.29**	5210.44*	0.01	5.48*	7.20	3625.69	2672.07	389.40	10619.27**	3.98E+08	4240.42*	14400315070	34741769.58
REC × Env	21	0.28	0.08	0.20	2676.79	0.01	3.34	5.87	2393.86	2698.86	481.55	6649.80*	4.65E+08*	2440.67	10692525117	23851658.45
Mat × Env	6	0.41	0.14	0.16	3036.06	0.01	0.98	1.93	3505.24	2169.33	800.45	3162.31	6.92E+08*	1736.07	4927431689	16887447.50
No mat × Env	15	0.23	0.05	0.21	2533.08	0.01	4.28	7.45	1949.30	2910.67	353.98	8044.80**	3.74E+08	2722.51	12998562488	26637342.83
Residual	192	0.41	0.14	0.15	3038.58	0.02	3.10	5.98	2734.83	1818.07	485.89	3482.07	2.68E+08	2624.45	11825782571	27729032.39
σ^2GCA/ σ^2SCA		0.73	0.02	0.46	0.06	0.36	0.01	-0.01	0.12	-0.14	0.10	0.03	0.00	-0.04	-0.01	-0.02
H^2		0.84	0.81	0.96	0.81	0.89	0.85	0.82	0.78	0.78	0.77	0.88	0.84	0.74	0.81	0.80
h^2		0.66	0.45	0.86	0.53	0.74	0.39	0.30	0.53	0.60	0.52	0.46	0.29	0.09	0.21	0.25

SCT7= *Striga* count at 7weeks, SCT10= *Striga* count at 10weeks, SDM7=*Striga* damage at 7 weeks, SDM10=*Striga* damage at 10 weeks, DTFT=Days to 50% tasselling, DTFS=Days to 50% Silking, DTFM=Days to 50% maturity, PLHT=Plant height, EHT=Ear height, CLHT=Cob length, CWPP= Cob weight/plant, GYPH=Grain yield/ha, HGWT=100 grain weight, SHGP=Shelling%, HIDX=Harvest index

tasselling, plant height, and ear height, suggesting that maternal gene effects exist in most characters, especially the Striga resistance characters. The maternal variance showed significance in only 8 characters. Striga damage at 10 weeks, days to 50% silking, days to 50% maturity, Number of seeds per row, and grain yield per plant were significant at p=0.01, while grain yield per hectare, *Striga* count, and *Striga* damage at 7 weeks were significant at 0.05% significance level. This suggests the presence of maternal gene effects on those characters which show significance in the infested environment. Additionally, the importance of maternal effects in certain traits emphasizes the need for careful selection of parental lines in breeding programs.

The variance component of all the interactions with the environment, were significant, both p=0.01 (Gen × Env., SCA × Env., Rec × Env and No Mat × Env) and p=0.05 (Mat × Env.) except GCA × Env. Both Gen × env. and Mat × Env were significant at 1% while SCA × Env and Rec × Env were significant at 5% for harvest index, suggesting the influence of environment on the performance of the genotypes. A similar trend was reported by Murtadha et al. (2018). Most mean interactions of the variance components with the environment for some characters were not significant, except for some *Striga*, yield and yield characters. Cob weight per plant and grain yield per hectare were significant at p=0.05 for Rec × Env. While grain yield per hectare and cob weight per plant were significant at 5% and 1% for Mat × Env and no mat × env respectively. This indicates the importance of the interaction of the genetic and environmental components in the resistance of *S. hermonthica* by the genotypes in the *Striga* inoculated pots. Similarly, a report has been published by Badu-Apraku et al. (2018) and Mohemed et al. (2016).

The broad sense heritability and narrow sense heritability estimates are also presented in Table 3. From the result, broad-sense heritability estimates were higher in magnitude (74% - 98%) for all characters are less than the narrow-sense heritability (9% - 88%). This is a result of the influence of non-additive gene action. The narrow sense heritability is most important in plant selection programs, as it captures only the proportion of genetic variation that is due to additive genetic value, which shows resemblance between relatives (Steinsaltz et al. 2020) . High, moderate, and low narrow-sense heritability were recorded, as also reported by Olaoye and Bello (2009).

Mean Performance of Parent

The mean performance of the parent varieties (*per se* performance) is presented in Table 4, with rankings based on their significance for *Striga* characteristics, earliness, growth, yield, and yield components. At week 7, Sammaz 16 recorded the lowest average *Striga* emergence/count, with only 0.08, while TZSR 193 had the highest count at 1.17. By week 10, TZSR 190 had the lowest *Striga* count, averaging 0.08, whereas Sammaz 16 exhibited the highest count at 1.33. In terms of *Striga* damage, TZE114 recorded the highest damage scores at both week 7 (2.42) and week 10 (4.17), while TZSR 190 showed the least *Striga* damage. With respect to characters that designate earliness among the parental lines, SUWAN took the longest number of days (55.33 days) to attain 50% tasseling while SAMMAZ 17 took the shortest days of 51.87 to attain 50% tasseling. SAMMAZ 16 had the highest day of 59.83 days to attain 50% silking while SAMMAZ 14 took the shortest days of 57.08 days to attain 50% silking SAMMAZ 17 took the longest days of 95.25 days to attain 50% maturity while TZSR 193 had the least days of 92.13 to attain 50% maturity.

With regard to the growth characters, SAMMAZ 17 was observed to be the tallest parent with a height of 183.18cm, while TZEI 114 and TZSR 190 were the shortest parents with 126.68cm and 128.42cm, respectively. A similar trend was also observed for ear height. SAMMAZ 16 exhibited the highest cob length (12.90) while TZSR 190 had the lowest cob length of 6.73cm.

SAMMAZ 16 equally showed to have the highest grain yield per hectare of 48.06g and 3.34tons per hectare, respectively, while TZSR 190 and TZEI 114 recorded the least values of 10.26g, 0.73tons, and 10.86 g, 0.77 tons, respectively, for both characters. SAMMAZ 16 also had the highest 100 grain weight (26.67g while TZEI 114 had the least of 10.51g. SAMMAZ 16 had the highest shelling percentage of 71.90 % while TZEI 114 and TZSR 190 recorded the least with 0.091 and 0.099, respectively.

Mean Performance of all the Genotypes

Table 4 presents the ranked mean values for the genotypes in this study. The findings indicate significant differences among all the genotypes (crosses and reciprocals derived from seven parents and three check varieties) across all measured traits. This suggests that the genotypes are suitable for selection procedures (Bahari et al., 2012; Fasahat et al., 2016).

At week 7, the cross TZSR 190 × SAMMAZ 16 had the lowest *Striga* count/emergence, with a value of 0.08, while the reciprocal cross SAMMAZ 14 × TZSR 193 recorded the highest *Striga* count of 2.58. In week 10, the reciprocal cross SUWAN × SAMMAZ 16 and the cross SAMMAZ 14 × SUWAN both had the lowest *Striga* count, each with a value of 0.33. The highest *Striga* count at week 10, 2.83, was observed in the reciprocal cross SAMMAZ 14 × TZSR 193.

At week 7, the reciprocal cross SAMMAZ 17 × TZSR 190 and the cross SAMMAZ 14 × SUWAN had the lowest *Striga* damage, both recording a value of 1.17, while the check GWG 111 showed the highest *Striga* damage at 3.00. By week 10, the reciprocal cross SAMMAZ 17 × TZSR 190 still had the least *Striga* damage rate at 1.58, whereas check 111 experienced the most damage, with a value of 4.42. Interestingly, some hybrids, such as TZSR 190 × SUWAN, with low *Striga* counts, were significantly damaged by *Striga* due to the subterranean germination of the parasites, which did not emerge at the surface. Conversely, hybrids like the reciprocal cross SAMMAZ 14 × TZSR 193 and SUWAN ×

TZSR 193, which had higher *Striga* counts, also suffered considerable damage due to their high susceptibility to *Striga* evasion. On the other hand, hybrids such as SAMMAZ 17 × TZSR 190, which had high Striga counts, showed little to no damage due to their tolerance to the parasite. Resistant hybrids, including SAMMAZ 14 × SUWAN, TZSR 190 × TZSR 193, TZSR 193 × TZSR 190, TZSR 190 × SAMMAZ 16, SAMMAZ 16 × TZSR 190, and check 5005, exhibited minimal Striga counts and damage. These hybrids also performed well in terms of yield and yield components. Similar trends were observed in previous studies by Gowda et al. (2021), Olakojo and Olaoye (2005), Sangaré et al. (2018), and Yallou et al. (2009). Kim (1994) emphasized that genotypes with low Striga counts but high Striga damage are not useful for breeding programs aimed at *Striga* resistance.

For characters of earliness, among all the genotypes (Crosses, reciprocals, and checks) evaluated, cross SAMMAZ 14 × SAMMAZ 17 recorded the shortest number of days (48.79 days) to attain 50% tasseling, while cross SAMMAZ 17 × SUWAN took the longest of 53.92 days to attain 50% tasseling. Similarly, reciprocal SAMMAZ 14 × TZSR 190 took the shortest number of days of 51.58 days, to attain 50% silking, whereas cross SAMMAZ 17 × SUWAN and SUWAN × TZEI 114 had the highest number of days of 57.75 days each, to attain 50% silking.

For days to 50% maturity, cross SAMMAZ 14 × SAMMAZ 17 had the least number of days of 84.54 days to attain 50% maturity, while reciprocal SUWAN × TZEI 114 had the longest days of 96.50 days to attain 50% maturity.

For growth characters, recip. SUWAN × SAMMAZ 14 recorded the highest plant height of 183.65cm at maturity, making it the tallest hybrid, while recip. SUWAN × TZSR 193 and check GWG 888 were the shortest among the hybrids, with 126.69cm and 128.90cm high, respectively. For cob length at maturity, check 5005 had the highest cob length of 15.67cm at maturity, while cross TZSR 190 × SAMMAZ 14 had the least cob length of 7.80cm.

From Table 4, it is observed that cross SAMMAZ 16 × SAMMAZ 17 had the highest grain yield in tons per ha of 51.50g and 3.56tons respectively, followed by cross TZEI 114 × SUWAN (50.45g and 3.52 tons), recip. SAMMAZ 16 × TZSR 193 with 48.52g and 3.34 tons, check 5005 with 48.19 and 3.33, SAMMAZ 14 × SUWAN with 47.46g and 3.29tons, TZEI 114 × SAMMAZ 16 46.41g and 3.23tons while recip. SAMMAZ 17 × SAMMAZ 16, check GWG 111 recorded the lowest yield per ha of 25.10g, 1.77tons and 26.39g, 1.89tons respectively. The highest shelling percentage of 73.20% was recorded in cross TZEI 114 × SAMMAZ 16, while check GWG 888 had the least shelling percentage of 49.30%. For harvest index, recip. SAMMAZ 16 × TZSR 190 had the highest harvest index of 0.271, while recip. SUWAN × TZSR 193 had the lowest harvest index of 0.131.

It is interesting to note that all the crosses that exhibited high performance in most characters and *Striga* resistance characters were a combination of one or two of the *Striga* resistance parental varieties, such as TZSR 190, SAMMAZ 16, and TZSR 193; however,

Table 4
Mean performance for Striga *resistance, growth, yield, and characters of forty-two hybrids (crosses and reciprocals), seven parents and three varieties of maize combined*

SOV	SCT7	SCT10	SDM7	SDM10	DTFT	DTFS	DTFM	PLHT	EHT	CLHT	CWPP	GYPH	HGWT	SHGP	HIDX
Genotype															
Parents															
TZSTR 190	0.25	0.08	1.33	1.33	54.17	59.53	93.58	128.42	56.84	6.25	14.07	0.73	17.44	49.30	0.091
TZSTR 193	1.17	0.42	1.92	2.33	52.50	58.33	92.13	148.74	68.87	7.83	31.53	1.68	20.09	66.70	0.143
TZEI 114	0.67	0.33	2.42	4.17	53.08	58.80	93.23	126.68	62.86	6.73	16.70	0.77	10.51	41.30	0.099
SAMMAZ 14	0.92	0.75	1.75	2.83	52.75	57.08	93.00	162.30	79.82	11.34	38.13	2.00	18.68	52.70	0.136
SAMMAZ 16	0.08	1.33	1.67	2.25	53.33	59.83	94.83	164.76	79.24	12.90	66.42	3.34	26.67	71.9	0.250
SAMMAZ 17	0.25	0.50	1.50	2.75	51.83	57.92	95.25	183.18	94.93	11.67	40.87	1.85	23.10	58.8	0.126
SUWAN	0.17	1.00	1.67	2.42	55.33a	59.50	95.17	170.26	79.38	11.46	47.89	2.40	22.28	67.10	0.181
Crosses															
TZSTR 190 × TZSTR 193	0.33	0.50	1.50	1.67	52.00	57.33	91.17	166.19	72.86	10.62	55.51	2.85	23.13	70.20	0.200
TZSTR 190 × TZEI 114	1.08	1.75	1.58	2.50	52.33	60.67	92.42	152.58	67.50	10.15	47.61	2.35	25.84	69.20	0.198
TZSTR 190 × SAMMAZ 14	0.83	0.67	1.67	2.92	52.58	57.71	89.83	164.19	81.31	7.8	42.77	2.27	20.80	61.60	0.186
TZSTR 190 × SAMMAZ 16	0.08	0.92	1.50	2.00	53.17	58.50	93.54	162.65	76.66	8.97	50.26	2.54	21.86	56.20	0.157
TZSTR 190 × SAMMAZ 17	1.08	0.50	2.00	2.25	53.50	58.93	93.05	177.97	89.10	9.29	60.12	2.80	23.31	59.80	0.163
TZSTR 190 × SUWAN	0.33	0.83	1.25	2.42	52.58	58.08	93.08	162.45	79.65	11.02	66.41	2.95	27.42	63.10	0.197
TZSTR 193 × TZEI 114	0.58	1.25	1.25	2.00	52.17	58.58	93.50	158.57	69.68	10.31	55.99	2.84	20.64	71.50	0.209
TZSTR 193 × SAMMAZ 14	0.25	1.42	1.50	2.58	52.92	58.92	93.25	157.36	72.67	12.21	52.42	2.45	22.09	66.40	0.190
TZSTR 193 × SAMMAZ 16	0.42	1.08	1.67	2.58	53.17	58.62	92.21	158.36	72.90	9.02	48.58	2.44	19.44	60.20	0.187
TZSTR 193 × SAMMAZ 17	0.83	0.67	1.58	3.25	52.17	61.33	92.67	154.36	72.50	9.81	53.31	2.72	22.54	71.70	0.209

Table 4 *(continue)*

SOV	SCT7	SCT10	SDM7	SDM10	DTFT	DTFS	DTFM	PLHT	EHT	CLHT	CWPP	GYPH	HGWT	SHGP	HIDX
TZSTR 193 × SUWAN	1.17	0.75	2.00	2.58	53.42	59.42	93.53	154.45	67.55	9.53	58.90	2.91	22.44	63.90	0.210
TZEI 114 × SAMMAZ 14	1.58	1.58	2.17	3.58	52.92	58.96	92.21	168.87	79.56	9.78	41.52	2.08	17.97	58.70	0.157
TZEI 114 × SAMMAZ 16	0.25	0.92	1.42	2.17	52.75	59.58	94.83	174.42	81.03	13.21	61.75	3.23	22.84	73.2a	0.224
TZEI 114 × SAMMAZ 17	0.42	1.33	1.67	2.67	52.83	58.83	93.33	167.57	80.18	11.17	50.76	2.41	21.80	69.00	0.192
TZEI 114 × SUWAN	1.08	1.00	1.75	2.75	52.08	57.83	93.08	177.48	82.16	12.58	77.39	3.52	24.13	67.20	0.242
SAMMAZ 14 × SAMMAZ 16	0.83	1.42	1.50	2.33	52.08	57.92	91.33	159.80	80.89	10.29	50.59	2.64	24.86	72.90	0.207
SAMMAZ 14 × SAMMAZ 17	1.67	1.25	2.42	3.92	52.58	57.58	90.67	145.93	73.19	10.13	54.86	2.73	19.65	71.10	0.234
SAMMAZ 14 × SUWAN	0.50	0.33	1.17	2.92	53.58	59.08	91.33	156.69	81.23	12.48	69.92	3.29	24.04	68.20	0.248
SAMMAZ 16 × SAMMAZ 17	0.67	0.67	2.00	3.25	52.67	57.50	90.67	157.43	79.19	10.64	71.50	3.56	22.34	68.11	0.223
SAMMAZ 16 × SUWAN	1.20	1.30	1.50	2.67	52.33	58.25	92.08	163.45	77.85	11.13	55.61	2.36	21.15	53.70	0.140
SAMMAZ 17 × SUWAN	0.17	1.00	1.75	2.50	52.67	57.83	98.17	166.29	79.78	10.84	45.50	2.10	24.52	55.80	0.143
Reciprocals															
TZSTR 193 × TZSTR 190	0.58	1.58	1.50	2.00	53.08	57.58	92.92	163.38	90.49	11.01	66.04	3.18	21.11	67.20	0.192
TZEI 114 × TZSTR 190	0.50	0.83	1.67	2.33	51.83	57.83	91.58	144.77	75.79	10.02	53.66	2.45	24.35	64.50	0.188
TZEI 114 × TZSTR 193	0.50	1.25	1.25	2.75	52.75	57.83	95.25	158.96	69.85	9.43	51.83	2.55	21.45	72.70	0.197
SAMMAZ 14 × TZSTR 190	1.08	1.33	2.08	3.08	52.00	59.42	93.33	155.90	78.52	9.18	44.09	2.18	23.79	64.50	0.182
SAMMAZ 14 × TZSTR 193	2.58	2.83	2.08	4.25	52.08	60.75	90.92	150.84	76.14	8.73	53.51	2.65	21.25	72.50	0.224

Table 4 *(continue)*

SOV	SCT7	SCT10	SDM7	SDM10	DTFT	DTFS	DTFM	PLHT	EHT	CLHT	CWPP	GYPH	HGWT	SHGP	HIDX
SAMMAZ 14 × TZEI 114	1.42	1.50	1.67	3.00	52.00	58.17	91.50	156.10	79.69	9.02	49.33	2.45	22.53	72.80	0.206
SAMMAZ 16 × TZSTR 190	1.33	1.33	1.58	1.83	52.92	58.83	94.42	168.59	86.00	11.01	58.74	3.03	25.06	71.80	0.271
SAMMAZ 16 × TZSTR 193	0.75	1.25	1.58	3.17	52.83	59.00	93.83	151.03	84.79	12.44	70.94	3.34	21.14	67.70	0.209
SAMMAZ 16 × TZEI 114	0.58	1.08	1.58	2.17	53.17	57.58	92.08	183.51	90.27	12.88	62.79	3.16	23.34	70.70	0.201
SAMMAZ 16 × SAMMAZ 14	1.75	0.75	2.33	3.75	53.08	59.17	93.17	150.81	71.18	11.01	55.53	2.65	20.64	57.90	0.166
SAMMAZ 17 × TZSTR 190	0.25	2.00	1.17	1.58	52.00	58.50	93.67	180.42	87.98	12.64	57.61	2.86	24.94	68.80	0.192
SAMMAZ 17 × TZSTR 193	0.92	2.17	1.58	2.83	51.33	56.79	92.08	155.91	79.83	10.70	50.02	2.37	18.79	56.20	0.160
SAMMAZ 17 × TZEI 114	1.08	0.83	1.67	2.50	53.50	58.92	98.83	160.18	74.34	12.78	53.46	2.71	23.35	71.30	0.197
SAMMAZ 17 × SAMMAZ 14	0.17	1.83	1.42	2.08	53.08	60.33	94.67	168.87	83.42	10.99	56.62	2.77	22.88	70.00	0.213
SAMMAZ 17 × SAMMAZ 16	1.08	1.08	1.83	3.33	52.75	57.92	94.35	143.08	75.97	8.68p	38.38	1.77	17.37	52.30	0.165
SUWAN × TZSTR 190	0.50	1.33	1.42	2.67	53.25	58.42	95.75	166.95	83.98	10.39	57.73	2.96	23.01	66.00	0.189
SUWAN × TZSTR 193	2.17	0.67	2.67	3.67	54.00	60.83	97.92	126.69	63.86	8.39	46.43	2.28	17.00	50.10	0.131
SUWAN × TZEI 114	0.42	1.25	2.17	3.42	53.92	58.67	94.88	175.95	78.81	12.06	5276	2.68	21.52	59.30	0.170
SUWAN × SAMMAZ 14	0.42	1.75	1.58	2.92	52.83	58.83	96.00	183.65a	86.83	12.18	62.61	3.19	25.62	70.00	0.172
SUWAN × SAMMAZ 16	0.50	0.33	1.50	2.25	52.67	59.67	96.75	177.76	82.98	13.18	58.20	2.85	23.14	66.30	0.183
SUWAN × SAMMAZ 17	1.08	2.75	2.42	2.92	54.00	61.92	94.17	143.10	89.17	8.93	39.07	2.06	16.30	48.10	0.134

Table 4 *(continue)*

SOV	SCT7	SCT10	SDM7	SDM10	DTFT	DTFS	DTFM	PLHT	EHT	CLHT	CWPP	GYPH	HGWT	SHGP	HIDX
Checks															
GWG111	0.58	0.92	3.00	4.42	53.00	59.29	96.71	158.93	68.08	9.89	38.86	1.89	19.46	58.50	0.156
5005	0.17	0.58	1.25	2.00	53.50	59.75	95.92	174.99	94.15	15.67	71.46	3.36	24.96	65.20	0.197
GWG888	0.67	1.33	2.17	4.17	52.25	59.58	92.33	128.90n	58.31	10.13	45.54	2.23	17.79	49.30	0.155
Mean	0.77	1.12	1.74	2.74	52.83	58.73	93.56	160.01	77.93	10.58	52.34	2.57	21.79	63.74	0.184
LSD	0.14	0.16	0.82	0.97	1.44	0.18	0.33	19.43	12.36	2.17	13.87	0.71	0.51	17.07	0.061
CV	236.29	203.69	77.78	85.01	4.95	6.88	4.76	22.76	28.02	42.95	56.21	59.20	39.57	35.54	48.47
											1.18				

SCT7= *Striga* count at 7weeks, SCT10= *Striga* count at 10weeks, SDM7=*Striga* damage at 7 weeks, SDM10=*Striga* damage at 10 weeks, DTFT=Days to 50% tasselling, DTFS=Days to 50% Silking, DTFM=Days to 50% maturity, PLHT=Plant height, EHT=Ear height, CLHT=Cob length, CWPP= Cob weight/plant, GYPH=Grain yield/ha, HGWT=100 grain weight, SHGP=Shelling%, HIDX=Harvest index

Table 5
General combining ability effect for the combined season for twenty-two characters

Parents	SCT7	SCT10	SDM7	SDM10	DTFT	DTFS	DTFM	PLHT	Non-In	EHT	CLHT	CWPP	GYPH	HGWT	SHGP	HIDX
TZSTR 190	-0.10	-0.08	-0.10*	-0.28**	0.00	0.01	0.38	-4.52	-3.42	-1.03	-3.18	-8.72	-141.82	-0.79	6540.24	-194.87
TZSTR 193	0.09	-0.01	0.01	0.01	-0.01	0.10	-0.19	4.62	-7.16**	-8.12	-1.86	1.13	-680.10	-1.61	13164.16	321.80
TZEI 114	0.01	0.01	0.03	0.07	0.01	-0.25*	-0.15	2.81	-3.79*	1.15	-2.34	1.18	716.21	0.01	-8965.63	307.10
SAMMAZ 14	0.17*	0.09	0.04	0.17**	-0.03	-0.25*	-0.20	-1.83	1.25	-2.80	-0.44	-7.17	-1733.83	-0.13	792.44	432.65
SAMMAZ 16	-0.07	-0.03	-0.01	-0.04	0.02	0.39*	0.28	5.47	3.75*	3.48	3.05	18.09*	3368.37	3.54	4958.05	317.09
SAMMAZ 17	-0.04	0.04	0.02	0.03	-0.03*	0.06	0.08	-11.24	6.81**	9.15	2.23	-3.50	-1524.39	2.42	-11014.16	-479.68
SUWAN	-0.06	-0.02	0.02	0.03fd	0.05**	-0.07	-0.20	4.69	2.57	-1.83	2.54	-1.00	-4.45	-3.44	-5475.10	-704.09

SCT7= *Striga* count at 7weeks, SCT10= *Striga* count at 10weeks, SDM7=*Striga* damage at 7 weeks, SDM10=*Striga* damage at 10 weeks, DTFT=Days to 50% tasselling, DTFS=Days to 50% Silking, DTFM=Days to 50% maturity, PLHT=Plant height, EHT=Ear height, CLHT=Cob length, CWPP= Cob weight/plant, GYPH=Grain yield/ha, HGWT=100 grain weight, SHGP=Shelling%, HIDX=Harvest index

some crosses that recorded high performance and *Striga* resistance had the combination of two *Striga* susceptible parents, such as TZEI 114, SUWAN, and SAMMAZ 14. The result of this present study also showed that most of the crosses that showed susceptibility were reciprocal hybrids, thereby suggesting that the choice of the donor and pollen receptor parents remains vital in the study of resistance to *Striga* in the maize plant. This is in agreement with the findings of Antoine et al. (2017). However, Kang et al. (1999), reported a contrary view in his work on where he asserted that the reciprocal effect is not important in the inheritance of rind puncture resistance in maize.

Genetic Component

General Combining Ability Effect for Striga *Resistance in Combined Season*

The estimates of general combining ability effects pooled over two seasons for all the characters are presented in Table 5. The results revealed TZSR 190 had the highest significant general combining ability estimate amongst the parental lines for traits where negative values are required like *Striga* damage and SAMMAZ 16 had the highest significant positive combining ability estimate where positive values are required like cob weight and other yield components. TZSR 190 also exhibited the highest negative value, though not significantly different from other parents for *Striga* count in both 7 and 10 weeks after planting, which is desirable. SAMMAZ 17 had the highest negative value for days to 50% tasselling, with SUWAN showing the highest positive significant value. TZEI 114 and SAMMAZ 14 had the highest significant negative GCA value of -0.25 each, while SAMMAZ 16 had the highest significant positive value of 0.39. It is interesting to note, however, that the parental varieties TZSR 190 and SAMMAZ 16 that performed well in *Striga* characters, yield, and their components are the best general combiners for *Striga* resistance, yield and yield components. Similar result was reported by Kim et al. (1998); Menkir (2006); Sangaré et al. (2018); Vivek et al. (2010) . Olaoye and Bello (2009), concluded that low GCA could probably be due to high tolerance of the parental varieties to *Striga* emergence.

Specific Combining Ability Effects of Crosses for Striga *Resistance in Season*

The specific combining ability effects of the crosses for *Striga* resistance characters, growth characters, yield and yield components for the 21 crosses in a glasshouse trial are presented in Table 6. Out of the 21 crosses, only two hybrid SAMMAZ 14 × SUWAN and TZSR 193 × TZEI 114 showed a negative SCA effect of -0.29 and -0.23 for *Striga* count at 7 weeks after planting. SAMMAZ 16 × SUWAN showed the highest non-significant negative SCA of -0.21 for *Striga* count at 10 weeks, while SAMMAZ 17 × SUWAN had the highest non-significant positive SCA value of 0.27. This shows that cross SAMMAZ 14 × SUWAN and TZSR 193 × TZEI 114 produce low strigolactones to stimulate *Striga* germination.

Similarly, TZSR 193 × TZEI 114 had the highest significant negative SCA (-0.29) for *Striga* damage at 7 weeks, followed by SMMAZ 14 × SUWAN (-0.26), while TZSR 193 × SUWAN had the highest significant positive SCA effects of 0.32. TZEI 114 × SAMMAZ 16 showed the highest significant negative SCA effect of -0.26, followed by TZSR 193 × TZEI 114 (-0.24), while SAMMAZ 16 × SAMMAZ 17 showed the highest highly significant positive SCA effect of 0.30 at 10 weeks. Crosses TZSR 193 × TZEI 114 and SAMMAZ 14 × SUWAN show high resistance to *Striga* infestation as they combine low *Striga* emergence and no visible *Striga* damage. Interestingly, the two crosses are a combination of a good general combiner (resistant) and a poor general combiner (susceptible) parental variety for *Striga* resistance. A similar finding was reported by Akaogu et al., (2017). TZSR 190 × TZEI 114 shows the only significant negative SCA effect of -0.07 for days to 50% tasselling while SAMMAZ 17 × SUWAN showed the highest highly significant negative SCA effects for both days to 50% silking and days to 50% maturity making the early flowering and maturity Cross hybrids which most desirable in maize especially in the savanna ecological region or areas with short rainfall. This is also in line with the reports by Akaogu et al. (2020) and Badu-Apraku et al. (2016).

From the result of Table 6, none of the crosses exhibited a significant SCA effect for plant height; however, TZSR 193 × SAMMAZ 16 had the highest negative SCA of -25.63, which makes it the shortest plant, which is desirable against lodging. TZSR 190 × SAMMAZ 17 had the highest significant positive SCA effects of 23.77 for ear height at maturity, preventing cob damage by rodents. This was also reported by Badu-Apraku et al. (2011). SAMMAZ 16 × SUWAN had the highest significant positive SCA of 13.90 for cob length at maturity. This cross hybrid may be considered in *Striga* free environment for high yielding performance, as cob length has been reported by Ahmad (2018) as a good attribute for yield in maize. The result of Table 6 also showed that in cob weight, TZSR 190 × SUWAN had the highest positive SCA effect of 34.91, while TZSR 193 × SUWAN exhibited the highest negative SCA effects (-39.47). SAMMAZ 14 × SUWAN showed the highest highly significant positive SCA effects of 14741.06 for grain yield per hectare and 100 grain weight (34.13), making it the best high yielding hybrid as it incorporates high grain weight, which is desirable for commercialization. This report affirms the findings of Amiruzzaman et al. (2013) and Ali et al. (2017). SAMMAZ 14 × SAMMAZ 17 had the highest highly significant positive SCA effects of 101769.91 and 4202.70 for shelling percentage and harvest index which also a desirable character in grain maize production as plant utilize assimilates for grain production whereas SAMMAZ 16 × SUWAN which had the highest highly significant negative SCA effects of -81701.81 for shelling percentage will be good for forage maize production for animal feeds. This finding is in agreement with the report by Aminu and Izge (2012).

Table 6
Specific combining ability effect for the combined season for twenty-two characters in maize

Crosses	SCT7	SCT10	SDM7	SDM10	DTFT	DTFS	DTFM	PLHT	EHT	CLHT	CWPP	GYPH	HGWT	SHGP	HIDX
TZSTR 190 × TZSTR 193	-0.20	0.04	-0.02	-0.14	-0.02	0.43	0.76	26.48	-11.09	5.33	25.38	4540.85	22.32	-3625.30	928.86
TZSTR 190 × TZEI 114	0.14	0.24	0.03	0.05	-0.07*	0.87	0.62	-15.17	-1.65	4.02	12.40	446.51	31.00*	33528.48	1282.53
TZSTR 190 × SAMMAZ 14	0.07	-0.09	0.13	0.21*	-0.02	-0.30	-0.77	2.29	0.80	-5.69	-10.28	-3965.35	-14.76	-32321.52	53.39
TZSTR 190 × SAMMAZ 16	0.13	0.08	0.01	-0.04	-0.02	-0.97*	-1.24	-10.74	-0.60	-0.63	-22.15	-882.58	-14.86	4060.07	-117.55
TZSTR 190 × SAMMAZ 17	0.02	0.16	0.00	-0.13	0.02	-0.06	-0.19	6.11	23.77*	2.57	25.88	9022.57*	11.11	14303.50	1507.31
TZSTR 190 × SUWAN	-0.07	0.09	-0.14	0.19	-0.02	0.63	0.73	-11.49	-11.04	7.34	34.91*	12667.30**	13.88	55480.92	1595.20
TZSTR 193 × TZEI 114	-0.23	0.06	-0.29**	-0.24*	0.02	0.70	1.22	-12.28	3.49	-1.58	7.41	7026.81	13.86	41073.11	2701.55*
TZSTR 193 × SAMMAZ 14	0.00	0.33	0.02	0.13	0.00	0.81	1.25	17.81	0.66	6.05	10.84	-5783.26	3.24	21315.33	974.88
TZSTR 193 × SAMMAZ 16	-0.09	0.07	-0.03	0.15	0.05	-0.42	-0.69	-25.63	8.20	-2.85	2.75	-570.26	-15.55	13567.90	-2218.20
TZSTR 193 × SAMMAZ 17	0.05	0.10	-0.11	0.12	-0.06	-0.77	-0.58	-9.34	1.36	-0.36	2.94	6607.28	-3.69	-17745.49	545.63
TZSTR 193 × SUWAN	0.47**	-0.19	0.32**	0.15	0.01	-1.12**	-1.64**	-10.13	-8.86	-4.61	-39.47**	-9377.66*	-21.84	-21013.84	-2014.67
TZEI 114 × SAMMAZ 14	0.25	0.09	0.06	0.02	-0.02	-0.13	-0.31	11.57	14.51	3.63	17.35	706.19	-18.07	-43135.16	-1877.11
TZEI 114 × SAMMAZ 16	-0.19	-0.01	-0.13	-0.26*	0.00	0.50	0.85	-8.14	6.17	4.91	12.53	12142.30**	8.79	55564.89*	2550.15
TZEI 114 × SAMMAZ 17	0.07	0.00	-0.06	-0.14	0.06	0.82	1.05	19.66	-14.49	5.60	4.71	-254.95	13.69	54340.07	1131.50
TZEI 114 × SUWAN	0.03	0.11	0.11	0.08	-0.03	-0.31	-0.19	18.48	-5.09	-4.22	32.58*	706.98	17.18	31633.72	-64.92
SAMMAZ 14 × SAMMAZ 16	0.15	-0.06	0.09	0.04	-0.05	0.51	0.75	-5.39	-14.18	-4.22	-11.19	-1918.40	13.32	-26671.56	937.76
SAMMAZ 14 × SAMMAZ 17	-0.01	0.12	0.05	-0.06	0.06	0.76	0.96	-21.41	-15.09	3.98	34.84*	5896.87	23.70	101769.91**	4202.70**
SAMMAZ 14 × SUWAN	-0.29	-0.04	-0.26**	-0.09	-0.01	0.91*	1.31*	6.70	2.50	5.23	23.23	14741.06**	34.13**	40520.07	1799.49
SAMMAZ 16 × SAMMAZ 17	0.15	-0.19	0.13	0.30**	-0.03	0.07	-0.24	7.28	-10.32	-4.71	-2.23	-9769.54*	-10.99	-42529.00	-1797.43
SAMMAZ 16 × SUWAN	0.17	-0.21	-0.12	-0.09	-0.04	0.34	0.21	19.24	6.94	13.90*	-35.08*	-5934.68	-4.45	-81701.81**	-1930.37
SAMMAZ 17 × SUWAN	-0.05	0.27	0.14	-0.08	0.01	-1.20**	-1.82**	-15.88	21.09	-11.23*	-35.04*	-7726.83	-32.04*	-39921.89	-2280.63

SCT7= *Striga* count at 7weeks, SCT10= *Striga* count at 10weeks, SDM7=*Striga* damage at 7 weeks, SDM10=*Striga* damage at 10 weeks, DTFT=Days to 50% tasselling, DTFS=Days to 50% Silking, DTFM=Days to 50% maturity, PLHT=Plant height, EHT=Ear height, CLHT=Cob length, CWPP= Cob weight/plant, GYPH=Grain yield/ha, HGWT=100 grain weight, SHGP=Shelling%, HIDX=Harvest index

Reciprocal Effect for Combine Analysis in Maize

An average of 7 hybrids showed a significant reciprocal effect value for *Striga* characters, as shown in Table 7. Hybrid SAMMAZ 17 × SAMMAZ 14 shows the highest significant negative recip. effect for *Striga* count at 7 weeks, while SAMMAZ 14 × TZSR 193 had the highest significant positive recip. Effect. TZEI 114 × TZSR 190 showed the highest significant negative recip. effect for *Striga* count at 10 weeks, while SUWAN × SAMMAZ 14 exhibited the highest significant positive recip. effect. For *Striga* damage at 7 and 10 weeks, SAMMAZ 17 × SAMMAZ 14 exhibited the highest significant negative recip. effect while SAMMAZ 16 × SAMMAZ 14 and SAMMAZ 14 × TZSR 193 had the highest significant positive recip. effect respectively.

From Table 7, SUWAN × SAMMAZ 16 has the highest significant negative recip. effect of -3.18 for days to 50% maturity, while SAMMAZ 14 × TZSR 190 has the highest positive significant recip. effect value of 1.56. For plant height at maturity, most of the hybrids showed negative recip. effect. SAMMAZ 16 × TZEI114 had the highest significant negative recip. value of -27.61, followed by SAMMAZ 16 × TZSR 193 with -20.26 while SUWAN × TZSR 190 exhibited the only significant positive recip. value of 21.85. For cob length, only SAMMAZ 16 × TZSR 193 and SAMMAZ 17 × TZSR 190 showed significant positive recip. effect of 14.00 and 7.08 respectively while SUWAN × TZEI 114 had the highest significant negative recip. effect of -15.38.

SAMMAZ 17 × TZSR 190 had the highest significant positive recip. of 12258.23 for grain yield per hectare, followed by SAMMAZ 16 × TZSR 190 (8915.56) and SAMMAZ 16 × TZSR 193 (8876.31) and SAMMAZ 14 × TZSR 190 (8362.71) while SUWAN × TZEI 114 had the highest significant negative recip. effect of -13121.18, followed by SUWAN × SAMMAZ 17 (-8997.10) and SAMMAZ 16 × SAMMAZ 14 with -8444.20. SUWAN × SAMMAZ 16 had the highest significant positive recip. value of 33.06 for 100 grain weight, followed by SAMMAZ 16 × TZSR 193 (23.56) and SAMMAZ 16 × TZSR 190 (23.24).

The above analysis showed that reciprocal hybrid SAMMAZ 17 × SAMMAZ 14, TZEI 114 × TZSR 190 and SAMMAZ 17 × TZSR 190 are good potential *Striga* resistant hybrids as they produce low strigolactone stimulant for *Striga* and non-visible *Striga* damage. It is interesting to note that most of the *Striga* resistant parental varieties are the donor parents in the hybrid combination, hence indicating that the choice of donor and receptor parents is vital in the development of *Striga* resistant hybrid. This is in line with the submissions of Chukwu et al. (2016) and Olaoye and Bello (2009).

In summary, this study provides valuable insights into the genetic mechanisms underlying Striga resistance, yield performance, and other agronomic traits in maize. The significant genotype-by-environment interactions highlight the need for location-specific breeding strategies, while the observed maternal effects and non-additive gene influences underscore the complexity of trait inheritance. The identification of high-yielding, Striga-

Table 7
Reciprocal effect for combined season in for twenty-two characters

Reciprocals	SCT7	SCT10	SDM7	SDM10	DTFT	DTFS	DTFM	PLHT	EHT	CLHT	CWPP	GYPH	HGWT	SHGP	HIDX
TZSTR 193 × TZSTR 190	0.05	0.34**	-0.01	0.09	0.01	-0.03	0.02	0.85	1.00	-1.69	-2.13	-195.51	-8.85	7403.85	681.31
TZEI 114 × TZSTR 190	-0.17	-0.31**	0.02	0.00*	-0.03	-0.11	-0.02	-2.00	8.10	3.67	2.45	894.94	4.34	8276.98	1538.62
TZEI 114 × TZSTR 193	-0.06	-0.04	-0.01	0.16	0.05	-0.06	0.01	-8.96	-4.22	-2.87	-22.99*	-2233.44	-0.30	-42693.69*	-264.99
SAMMAZ 14 × TZSTR 190	0.10	0.23*	0.11	0.02	-0.07**	1.27**	1.56**	-9.52	-18.45*	4.69	1.87	8362.71**	15.83	22978.20	1394.46
SAMMAZ 14 × TZSTR 193	0.59**	0.13	0.17*	0.35**	-0.04	0.00	-0.09	-17.80	-8.14	-7.82*	-25.24*	-4492.40	-15.33	7130.55	-542.64
SAMMAZ 14 × TZEI 114	-0.04	-0.14	-0.12	-0.14	-0.03	1.14**	1.49**	-15.48	-37.30**	-1.47	39.42**	3272.81	9.51	41588.56*	565.41
SAMMAZ 16 × TZSTR 190	0.42**	0.11	0.04	-0.05	-0.03	1.12**	1.48**	-0.24	20.64**	7.08	40.20**	8915.56**	23.24*	72815.62**	4428.81**
SAMMAZ 16 × TZSTR 193	0.13	0.07	-0.02	0.14	0.02	0.61*	1.52**	-20.26*	-1.85	14.00**	54.31**	8876.31**	23.56*	39527.68*	1776.15*
SAMMAZ 16 × TZEI 114	0.11	0.02	0.06	0.03	-0.02	-0.08	-0.09	-27.61**	1.30	0.07	30.21*	5478.83	-4.09	-4878.42	-1295.46
SAMMAZ 16 × SAMMAZ 14	0.27*	-0.21	0.22**	0.30**	0.07**	0.06	-0.01	6.62	-6.86	-1.70	-19.43	-8444.20**	-23.60*	-45824.91*	-1866.91*
SAMMAZ 17 × TZSTR 190	-0.17	0.46**	-0.23**	-0.17*	-0.04	0.62*	0.79	-17.29	-10.95	7.71*	7.95	12258.23**	3.95	37066.27*	2129.67*
SAMMAZ 17 × TZSTR 193	0.05	0.36**	0.01	-0.11	-0.06*	-1.30**	-1.51**	17.65	0.64	1.42	-15.22	-4185.46	-14.82	-29778.76	-1048.71
SAMMAZ 17 × TZEI 114	0.22*	-0.07	0.01	-0.03	0.04	0.03	0.08	-11.25	18.94*	-6.77	20.21	2724.79	-8.44	2237.40	-1384.77
SAMMAZ 17 × SAMMAZ 14	-0.49**	0.17	-0.26**	-0.40**	-0.01	0.05	0.07	-15.59	4.46	5.76	-1.47	-2503.71	4.47	-3876.08	-724.81
SAMMAZ 17 × SAMMAZ 16	0.06	0.22	-0.05	0.01	-0.01	0.04	-0.67	-19.37*	9.88	-10.36*	-43.59**	-6449.02*	-35.84**	-4351.04	-3161.59**
SUWAN × TZSTR 190	0.04	0.16	0.04	0.05	-0.01	-0.05	-0.02	21.85*	-6.98	-4.46	-14.54	1091.67	1.19	-6680.68	-1316.11
SUWAN × TZSTR 193	0.25*	-0.08	0.19**	0.23**	0.01	-0.57	-1.45**	-6.91	15.41*	-10.46*	-27.54*	-7900.61**	-30.75**	-47552.55*	-2856.59**
SUWAN × TZEI 114	-0.17	0.13	0.14*	0.15*	0.00	-1.21**	-1.53**	-19.78*	-14.32*	-15.38**	-23.35*	-13121.18**	-21.34*	-32795.91	-1404.79
SUWAN × SAMMAZ 14	-0.03	0.49**	0.11	0.00	-0.03	0.00	0.08	4.51	16.34*	-4.71	10.54	-2492.96	7.72	11722.56	-166.25
SUWAN × SAMMAZ 16	-0.17	-0.19	0.00	-0.08	0.00	0.10	0.89*	-5.81	-16.51*	5.07	43.63**	8046.14**	33.06**	54053.05**	2375.99*
SUWAN × SAMMAZ 17	0.25*	0.41**	0.14*	0.07	0.03	-1.67**	-3.18**	14.59	2.59	-10.43*	-33.74**	-8997.10**	-48.76**	-42954.50*	-3324.64**

SCT7= *Striga* count at 7weeks, SCT10= *Striga* count at 10weeks, SDM7=*Striga* damage at 7 weeks, SDM10=*Striga* damage at 10 weeks, DTFT=Days to 50% tasselling, DTFS=Days to 50% Silking, DTFM=Days to 50% maturity, PLHT=Plant height, EHT=Ear height, CLHT=Cob length, CWPP= Cob weight/plant, GYPH=Grain yield/ha, HGWT=100 grain weight, SHGP=Shelling%, HIDX=Harvest index

resistant hybrids offers a pathway for improving maize productivity in *Striga* endemic regions. These findings have important implications for maize breeding programs, as they provide a scientific basis for selecting superior genotypes, optimizing hybrid combinations, and developing resilient maize varieties suited for diverse environmental conditions.

CONCLUSION

In conclusion, the identification of hybrids with high Striga resistance and desirable agronomic traits has practical implications for improving food security in Striga-prone regions. Farmers can benefit from hybrids like TZSR 190 × SAMMAZ 16 and SAMMAZ 14 × SUWAN among others which combine Striga resistance with high yield potential. Moreover, hybrids with early maturity characters, such as SAMMAZ 14 × SAMMAZ 17, are particularly valuable for regions with short growing seasons. In addition, since *Striga* resistance genes are controlled by recessive genes, which could be cytoplasmic or non-nuclear genes as revealed in this study, identifying the donor and pollen receptor will be essential in the breeding program for *Striga* resistance. These findings can guide researchers, seed companies, and agricultural extension services in recommending suitable varieties to farmers in *Striga* endemic regions.

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Optimization of Blending Ratio of Seasoned Flour from Modified Cassava Flour, Sago Flour, and Cornstarch by D-Optimal Mixture Design Approach

Raden Cecep Erwan Andriansyah[1], Taufik Rahman[2]*, Chandra Maulana[3] and Ela Turmala[3]

[1]*Research Center for Food Technology and Processing, National Research and Innovation Agency, Gunung Kidul 55861, Indonesia*
[2]*Research Center for Appropriate Technology, National Research and Innovation Agency, Tangerang Selatan 15314, Indonesia*
[3]*Department of Food Technology, Faculty of Engineering, Universitas Pasundan, Bandung 40153, Indonesia*

ABSTRACT

This study aimed to investigate the optimum ratio between modified cassava flour, sago flour, and cornstarch in order to improve the physicochemical and sensory qualities of seasoned flour. To achieve this objective, the D-optimal method in Design Expert software was used to create 16 formulas. The respective ranges for modified cassava flour, sago flour, and cornstarch were 37.5%–42.5%, 27.6%–32.5%, and 16.0%–20.0%. Furthermore, moisture content, water holding capacity (WHC), oil holding capacity (OHC), and sensory acceptance were analyzed. With a desirability value of 0.774, the predictive results showed that the optimal composition of 41% modified cassava flour, 31% sago flour, and 16% cornstarch produced the best results. According to physicochemical and functional analysis, the optimum seasoned flour formula had 8.06% moisture content, 6.32% ash, 2.67% protein, 0.40% fat, 80.31% carbohydrate, 5.01% resistant starch, 18.52% WHC, and 10.20% OHC.

Keywords: Autoclaving-cooling cycle, mixture design, modified cassava flour, optimization, seasoned flour

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E-mail addresses:
rade015@brin.go.id (Raden Cecep Erwan Andriansyah)
tkr.ttg@gmail.com (Taufik Rahman)
cm.chandramaulana@gmail.com (Chandra Maulana)
elaturmala@unpas.ac.id (Ela Turmala)
*Corresponding author

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INTRODUCTION

Seasoned flour is a food ingredient made from a mixture of flour and spices. Commercially available seasoned flour is generally made from composite flour, salt, spices, and other food additives. Meanwhile, packaged seasoned flour is often used for fried chicken or other fried food items as it saves cooking time. According to Isaskar et al. (2021), consumers prefer seasoned flour made from modified cassava flour to that made from wheat flour.

Composite flour was used as the base material for seasoned flour in this study. It is made from flours of cereals (corn, rice, and sorghum), tubers (cassava, sweet potatoes, and potatoes), which are high in carbohydrates, and legumes (soybeans), rich in protein. These ingredients may be used either alone or in combination with wheat flour (Seibel, 2006). Compared to wheat flour, composite flour offers higher protein content and more vitamins, making it a more nutritious option (Olaoye et al., 2006). The unique color, texture, and nutritional content of each component of composite flour can be considered a culinary advantage. Currently, composite flour is used in a wide variety of recipes around the world, particularly for cakes, bread, pastries, noodles, and pasta (Engindeniz & Bolatova, 2019).

A form of tuber potentially used as a functional food ingredient is cassava (*Manihot esculenta*), which can be modified to improve the functional properties of native starch. Native starch is considered negligible as the physical and chemical characteristics make it unsuitable for general use. A special treatment applied to starch to improve or alter certain properties is known as starch modification (Aparicio-Saguilán et al., 2005). In this study, cassava flour was used and modified using the autoclaving-cooling cycles method. The autoclaving-cooling cycles process involves a combination of heating using an autoclave, resulting in the complete gelatinization of starch (with the amylose fraction leaching out of the granules), followed by a low-temperature storage process that increases the retrogradation of starch through amylose crystallization (Sajilata et al., 2006). The modification process, involving repeated autoclaving-cooling cycles, increases the arrangement of amylose-amylose and amylose-amylopectin interactions and improves the formation of more perfect crystals, leading to higher levels of type 3 resistant starch (Leong et al., 2007). Cassava flour modified by autoclaving-cooling cycles has 6.44% moisture content, 1.78% ash, 2.76% protein, 0.10% fat, 85.34% carbohydrate, 67.31% starch, 24.22% amylose, 43.08% amylopectin, 4.45% resistant starch, 59.31 (%WT) white degree, 28.85% water holding capacity (WHC), and 10.20% oil holding capacity (OHC) (Rahman et al., 2017).

Corn (*Zea mays*), the third-most important grain crop in the world after rice and wheat, is processed to produce a wide range of food and industrial products, including starch, sugars, oils, beverages, and biofuels (Yu & Moon, 2022). A vital component often used in the food industry is cornstarch, which serves as a thickening, bulking, gelling, and water retention agent, as well as a colloid stabilizer (Singh et al., 2003). Even though excessive use of cornstarch can make fried food overly dense, it is known to improve crispiness when frying and can cause the food to break more easily when bitten (Perera & Embuscado, 2014).

Sago palm (*Metroxylon* spp.) is an important plant, particularly for the people of Southeast Asia and its surrounding regions (Indonesia, Malaysia, and Papua New Guinea), due to its wide range of uses (Konuma, 2018). Sago flour, processed from the tree trunks, has a competitive advantage in both yield and price compared to corn and cassava starch (Du et al., 2020). The composition is approximately 88% carbohydrates, with 21.4%–30.0% being amylose (Martinez et al., 2018). It has a higher swelling ability, lower enzyme digestibility than cornstarch, higher gelatinization temperature, and lower peak viscosity, thereby useful as a stabilizer (Achudan et al., 2020; Karim et al., 2008).

This study used modified cassava flour, sago flour, and cornstarch as raw materials for making seasoned flour. These ingredients are abundantly available to the population in the study area. The differences in their properties and characteristics necessitate formula optimization to produce seasoned flour with good nutritional content, physical properties, and sensory acceptance. Design Expert is an effective method for optimizing the combination of test materials, requiring fewer resources than traditional methods (Zhong et al., 2007). One of the design options provided by Design Expert is D-optimal mixture, which aims to find the optimal formulation.

Previous studies on the development of seasoned flour used sword bean flour, wheat, and sago (Purwaningsih et al., 2021); fermented soybean flour and roasted *Moringa oleifera* seed flour (Omeje et al., 2021); sweet potato, rice, and tapioca flours (Alfani et al., 2019); corn flour (Christian et al., 2022); as well as jack bean flour, wheat flour, and sago flour (Purwaningsih et al., 2024). The development of seasoned flour using cassava flour, sago flour, and cornstarch is promising due to the ability to increase the use of local raw materials. Only a few studies have focused on the functional properties of flour and seasoned flour made from modified cassava flour, sago flour, and cornstarch (Rahman et al., 2017. This current study provided information on composite flour materials to increase the nutritional content of seasoned flour. Due to the limited investigations on seasoned flour formulation using optimization methods to obtain the optimal formulation, this study aimed to optimize the ratio of modified components to produce seasoned flour with improved nutritional value, as well as better physical and sensory properties.

MATERIALS AND METHODS

Materials

Cassava var. Manggu (*Manihot esculenta*), obtained at approximately 9 months of age from Tanjung Siang District, Subang Regency, served as the raw material for this experiment. Cornstarch, sago flour, baking soda, salt, pepper powder, garlic powder, flavoring, cooking oil, and chicken fillet were purchased from a local market in Subang Regency, Indonesia.

Seasoned Flour Preparation

The autoclaving-cooling cycle method was used to prepare modified cassava flour (Andriansyah et al., 2017). The first step involved weighing the materials according to the formulation. The ingredients (modified cassava flour, sago flour, cornstarch, and spices) were stirred until homogeneous, and the seasoned flour mixture was subsequently applied as a coating on fried chicken.

Chicken meat was cut into pieces weighing 5-6 grams. A wet batter for fried chicken, seasoned flour was prepared with a 25:75 ratio of flour to water. Chicken was coated by first dipping into the dry seasoned flour, then in the wet batter, followed by draining, and finally dipping back in the dry seasoned flour. The next step involved deep-fat frying at a temperature of 170°C for 5 minutes. The final stage was draining the oil.

Data Collection and Analysis

Sensory Evaluation

Evaluation was conducted hedonically on color, taste, texture, aroma, and overall acceptance, with a numerical assessment on a scale of 1–6, where 1 = very dislike, 2 = dislike, 3 = slightly dislike, 4 = slightly like, 5 = like, and 6 = very like. A total of 30 semi-trained panelists were selected and briefed on the six-point hedonic scale and its use prior to the assessment. Fried chicken was prepared, tossed with seasoned flour, placed on a white platter, and randomly coded. Subsequently, panelists were instructed to sip water between each sample test during the evaluation. Data from the sensory evaluation were analyzed using Analysis of Variance (ANOVA), and when significant, the Duncan test was applied.

Chemical Properties Analysis

The gravimetric method was used to determine the sample's moisture and ash content. Protein content was determined using the Kjeldahl method for crude protein analysis, with a conversion factor of 6.25 according to AOAC method 2001.11 (AOAC, 2005). Crude fat was determined by the Soxhlet extraction method according to AOAC method 920.39 (AOAC, 2005). Meanwhile, total carbohydrate content was determined using the "by difference" method.

Resistant Starch Analysis

Resistant starch was analyzed using a method from Kim et al. (2003) with minor modifications. An Erlenmeyer flask containing 0.5 grams of material was filled with 25 milliliters of 0.1 M pH 7 phosphate buffer solution, which was swirled to create a suspension. The sample was placed in the flask, and 0.1 milliliters of alpha-amylase enzyme was added. The Erlenmeyer flask was subsequently covered with aluminum foil and incubated for 15 minutes at 100°C in a water bath, with periodic stirring. After removal and cooling of the sample, 5 mL of 1 N HCl and 20 mL of distilled water were added. The flask was filled with 1 milliliter of 1% pepsin enzyme, covered, and heated to 40°C in a water bath for one hour. After removal, 5 mL of 1 N NaOH, distilled water, and 0.1 mL of beta-amylase enzyme were added to the flask. After sealing, the Erlenmeyer flask was incubated for one hour at 40°C in a shaking water bath. The mixture was filtered through filter paper, the residue was dissolved, and the starch content was determined.

Physical Properties

WHC and OHC were determined using methods from Adebiyi and Aluko (2011), with minor modifications. A 250 mg sample was dissolved in 5 mL of distilled water (or cooking oil). The suspension was kept at room temperature for 15 minutes, with shaking every 5 minutes. Furthermore, it was centrifuged for 15 minutes at 3,000 rpm. The volume of the separated distilled water (or separated cooking oil) was measured, and WHC (or OHC) value was calculated by dividing the volume of sediment by the volume of the suspension.

Seasoned Flour Formula Optimization

After determining the variables to be mixed and their concentrations, the response to be measured was defined based on the product's components. Each response variable was analyzed using the Design Expert application to determine the D-optimal equation, which could be linear, quadratic, or cubic. These variables were used as a prediction model to ascertain the ideal formula. After processing each response variable according to the specified criteria, Design Expert provided solutions for several potential ideal formula responses. Desirability, a value between 0 and 1, was used to represent the target optimization value achieved. The closer the reaction to the value "1," the closer the formula is to the ideal point. Optimization was carried out to maximize the desirability value. Although the primary objective of formula optimization was to find the ideal mixture of various material combinations, achieving a desirability value of 1 was not the ultimate aim.

The lower and upper limits were selected using sensory tests to determine the formula to be optimized. According to Rahman et al. (2017), the sample selected based on sensory tests had a ratio of 40% modified cassava flour, 30% sago flour, and 18% cornstarch. These results were used to establish the upper and lower limits for the amounts of modified cassava flour, sago flour, and cornstarch, ensuring that the formulation was both optimized and met the required standards, as determined using the Design Expert program. The upper and lower limit data are presented in Table 1.

Table 1
Lower and upper limit data variable change

Variable Changed	Lower limit (%)	Upper limit (%)
Modified cassava flour	37.5	42.5
Sago flour	27.5	32.5
Cornstarch	16.0	20.0

In the preparation of seasoned flour, other ingredients were used as fixed variables and added to the dough. The concentration of these variables was not included in the experimental design. The ingredients included powdered spices (3% salt, 3% pepper powder, 2.5% garlic, 1% coriander, and 1.5% flavoring) and food additives (1% baking soda). The proportions of these additional ingredients were considered separately from the variable ingredients (modified cassava flour, sago flour, and cornstarch). The primary tool used to determine the optimal formulation and the relative percentage of each flour was Design Expert mixture design program with the D-optimal model.

RESULTS AND DISCUSSION

Determination of Seasoned Flour Formula Design

Based on the Design Expert program, 11 variations of the total flour composition were obtained without grouping, as part of an experimental design with 5 repetitions, resulting in 16 models of seasoned flour, as shown in Table 2. The results of physicochemical and sensory analysis of seasoned flour are presented in Table 3.

Table 2
Optimization design of seasoned flour formula using Design Expert

Formula	Modified cassava flour (%)	Sago flour (%)	Cornstarch (%)
1	40.0	29.9	18.0
2	38.9	29.1	20.0
3	42.3	29.7	16.0
4	42.5	27.7	17.8
5	37.5	30.8	19.7
6	38.9	32.5	16.6
7	38.9	32.5	16.6
8	40.4	27.6	20.0
9	42.5	27.7	17.8
10	40.7	31.3	16.0
11	40.4	27.6	20.0
12	38.7	31.0	18.3
13	37.5	32.4	18.1
14	41.0	28.5	18.5
15	37.5	30.8	19.7
16	42.3	29.7	16.0

Table 3
Input data from the response analysis results in Design Expert

Formula	Modified cassava flour (%)	Sago flour (%)	Corn starch (%)	OHC (%)	WHC (%)	Moisture content (%)	Sensory analysis				
							Color	Taste	Texture	Aroma	Over All
1	40.0	29.9	18.0	11.50	18.87	7.90	4.5	4.7	4.0	4.7	4.6
2	38.9	29.1	20.0	10.00	17.31	7.81	4.4	4.4	4.2	4.4	4.5
3	42.3	29.7	16.0	11.54	17.31	8.10	4.6	4.7	4.3	4.7	4.8
4	42.5	27.7	17.8	13.72	19.23	8.17	4.2	4.5	3.8	4.8	4.6
5	37.5	30.8	19.7	9.80	16.98	8.18	4.2	4.7	4.2	4.7	4.8
6	38.9	32.5	16.6	13.46	17.31	8.15	4.2	4.7	4.2	4.7	4.7
7	38.9	32.5	16.6	11.76	16.67	8.15	4.2	4.6	4.2	4.6	4.7
8	40.4	27.6	20.0	11.54	18.87	8.11	4.6	4.6	4.5	4.7	4.9
9	42.5	27.7	17.8	13.72	19.23	8.07	4.2	4.6	4.5	4.5	4.7
10	40.7	31.3	16.0	11.76	18.87	7.81	4.6	4.8	4.8	4.8	5.2
11	40.4	27.6	20.0	10.00	18.87	7.72	4.7	4.8	4.6	4.7	5.2
12	38.7	31.0	18.3	10.00	18.87	7.78	4.4	4.8	4.7	4.6	4.9
13	37.5	32.4	18.1	10.00	18.52	7.90	4.5	4.8	3.8	4.7	4.8
14	41.0	28.5	18.5	12.00	19.23	7.78	4.3	4.9	4.4	4.7	4.8
15	37.5	30.8	19.7	9.80	16.98	8.04	4.4	4.7	3.9	4.7	4.8
16	42.3	29.7	16.0	10.00	19.23	8.08	4.5	4.8	4.0	4.6	4.7

Water and Oil Holding Capacity

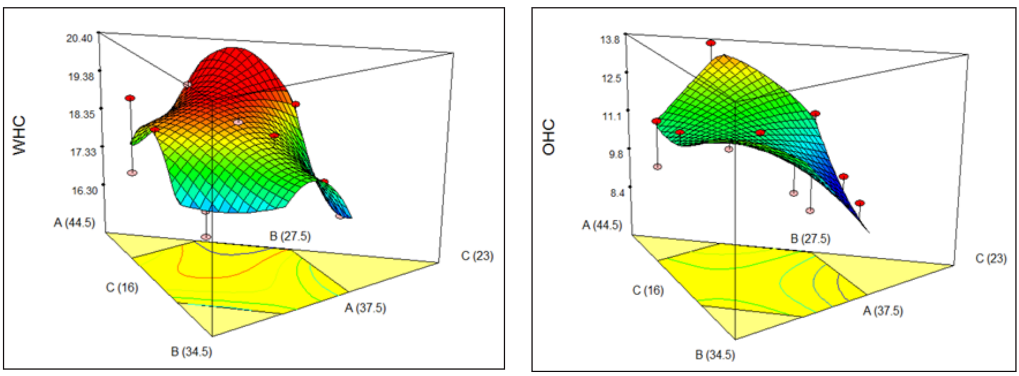
The ability of flour to retain water absorbed was measured by WHC. Smaller starch granules increased solubility and water absorption in flour, which was influenced by size and shape (Niba et al., 2001). The analysis showed that seasoned flour had WHC ranging from 16.67% to 19.23%. WHC value of this seasoned flour was lower compared to 22.06%–31.20% obtained from Herminiati et al. (2017). The results of the ANOVA test showed that the recommended model was a special cubic. The polynomial equation for WHC response is presented as follows.

$$\text{Water holding capacity (\%)}: Y = 13.25A + 12.14B + 4.17C + 24.42AB + 44.18AC + 37.85BC - 74.62ABC$$

Where:
A=Modified cassava flour
B=Sago flour
C=Cornstarch

Adding modified cassava flour, sago flour, and cornstarch significantly affected WHC of seasoned flour. Adding each flour component separately, as well as the two-component interactions (AB, AC, and BC), increased WHC value, confirmed by positive constant values. However, the interaction between the three components resulted in a decrease in WHC. Adding modified cassava flour had the most significant influence on increasing WHC, as it had the highest constant value.

A three-dimensional graph illustrating the interactions between the components is shown in Figure 1. Different response values are represented for each combination of formula components, based on the variation in surface height. The high areas corresponded to higher WHC response values, while the low areas corresponded to lower values.



(a) (b)

Figure 1. Three-dimensional graph of WHC and OHC: (a) WHC; (b) OHC

The ability of flour to retain the oil absorbed was measured by OHC. According to Adebowale et al. (2005), the ability to bind oil is influenced by the lipophilic groups coating the starch granules. The fat contents in starch, both in the granules and those covering the granules, created a hydrophobic surface, helping to bind oil components or other fats added externally. Therefore, the fat absorption capacity was influenced by the fat components in the starch granules. Flour with a high OHC value could absorb and retain more oil during frying. OHC measurements ranged from 9.8% to 13.72%. OHC value of this seasoned flour was lower compared to the 16.33% to 18.05% obtained by Herminiati et al. (2017). ANOVA test showed that the model for OHC response was quadratic. The polynomial equation for OHC response is presented as follows.

$$\text{Oil holding capacity (\%)}: Y = 12.85A + 14.01B + 3.85C - 8.59AB + 15.01AC + 2.08BC$$

Where:
A=Modified cassava flour
B=Sago flour
C=Cornstarch

Adding modified cassava flour, sago flour, and cornstarch significantly affected OHC of seasoned flour. Adding each flour component separately, as well as the two-component interactions (AC and BC), increased OHC, as confirmed by positive constant values. However, the interaction between AB components (sago flour and cornstarch) decreased OHC. Since sago flour had the highest constant value, its addition had a significant impact on increasing OHC.

Moisture Content

Products made with flour of high moisture content tended to clump and were more susceptible to microbial deterioration. In seasoned flour samples measured in this study, the moisture content ranged from 7.72% to 8.18%. This was lower than the moisture content of a composite of wheat, rice, green gram, and potato flour, ranging from 10.93% to 11.67% (Chandra et al., 2015). ANOVA test showed that the recommended model was quadratic. The polynomial equation for the moisture content response is presented as follows.

Moisture content (%): $Y = 8.71\ A + 8.57B + 8.50C - 2.70AB - 2.67AC - 2.04BC$

Where:
A=Modified cassava flour
B=Sago flour
C=Cornstarch

The analysis showed that the model was not statistically significant. The addition of modified cassava flour, sago flour, and cornstarch did not significantly affect the moisture content, likely due to their similar particle sizes and homogeneous mixing, which minimized variation in water-binding capacity. Adding each flour component separately increased moisture content, confirmed by positive constant values, while the interaction between components AB, AC, and BC decreased the moisture content. The polynomial model recommended by the Design Expert Program is a two-factorial interaction, but this model shows a very small Predicted R-Squared value, so it is necessary to do model reduction using backward elimination. Model reduction is done to eliminate the interaction of raw material components, because they are considered insignificant (do not meet $\alpha out = 0.1000$). Furthermore, the addition of modified cassava flour had the most significant influence on the increase in moisture content, as it had the highest constant value. A three-dimensional graph of moisture content response test results is presented in Figure 2.

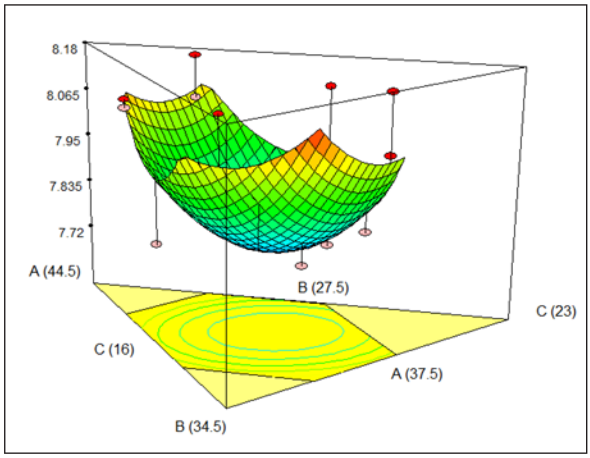


Figure 2. Three-dimensional graph of moisture content response

Sensory Evaluation

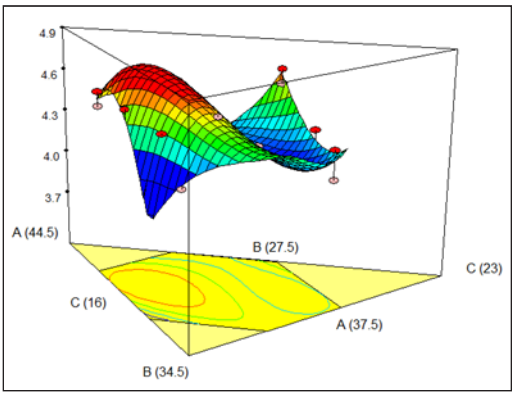
The results of the color response measurement produced values ranging from 4.2 to 4.7, showing that the panelists rated the color of the seasoned flour product as "slightly like" to "like." Based on the model analysis, the color response was cubic. The polynomial equation for the color response is presented as follows.

$$\text{Color: } Y = 2.86A + 2.30B + 11.64C + 8.09AB - 11.30AC - 10.60BC + 9.41ABC + 1.89AB(A-B) + 12.11AC(A-C) + 18.64BC(B-C)$$

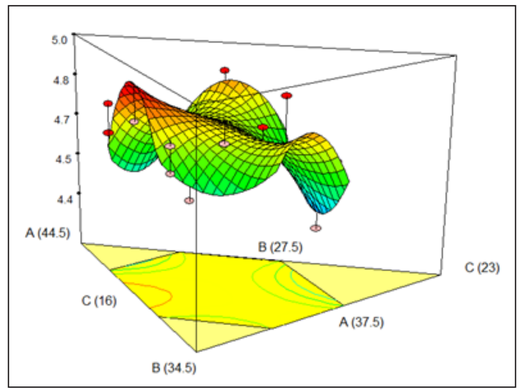
Where:
A=Modified cassava flour
B=Sago flour
C=Cornstarch

The color response of seasoned flour was significantly affected by the addition of modified cassava flour, sago flour, and cornstarch. Adding each flour component separately, as well as the interaction between all three components (ABC), increased the color value, confirmed by a positive constant. However, the interaction between AC and BC components decreased the color value. The addition of cornstarch had the most significant influence on the increase in color value, as it had the highest constant value. Fried chicken products had a golden-brown color due to a non-enzymatic browning reaction, specifically the Maillard reaction.

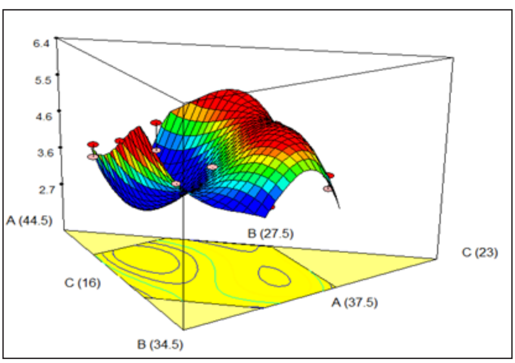
Figure 3 shows the three-dimensional graph of the sensory evaluation and the interaction between the components. For each combination of formula components, the variation in surface height represented a different response value. The high areas corresponded to higher response values, while the low areas corresponded to lower response values.



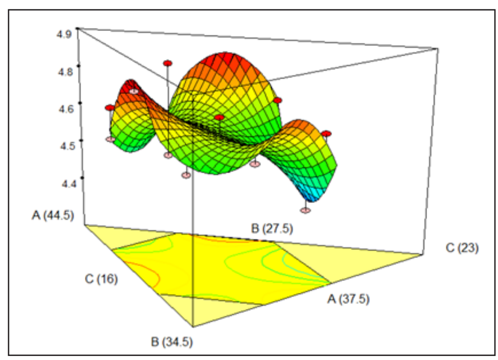
(a)



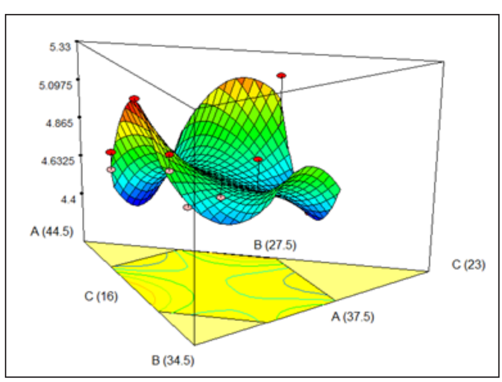
(b)



(c)



(d)



(e)

Figure 3. Three-dimensional graph of sensory evaluation: (a) color; (b) taste; (c) texture; (d) aroma; (e) overall

The results of the taste response measurement produced values ranging from 4.4 to 4.9. Fried chicken, coated with seasoned flour, tasted savory. Sensory acceptance value showed that panelists tended to rate the taste of fried chicken seasoned flour products more favorably. Based on the model analysis, the taste response was a special cubic. The polynomial equation for the taste response is presented as follows.

$$\text{Taste: } Y = 3.43A + 3.68B + 2.38C + 5.47AB + 7.62AC + 6.90BC - 17.16ABC$$

Where:
A=Modified cassava flour
B=Sago flour
C=Cornstarch

ANOVA test showed that the taste response of seasoned flour was not significantly affected by the addition of modified cassava flour, sago flour, or cornstarch. Adding each flour component separately increased the taste value, confirmed by a positive constant. However, the interaction between ABC components decreased the taste value. The increase in taste value was most significantly influenced by the addition of sago flour, which had the highest constant value.

Texture is a parameter that can be evaluated through the sense of taste (mouthfeel) or touch (handfeel). In this study, panelists only evaluated mouthfeel. In coated fried products, crispiness was influenced by the ability of the coating flour to absorb and retain water. Texture is closely related to a product's crispiness, a sensation associated with detecting small cracks in the mouth, often accompanied by the sound of food cracking or breaking (Van Vliet et al., 2007).

$$\text{Texture: } Y = 8.52A + 3.51B - 29.57C - 5.81AB + 63.28AC + 69.17BC - 103.04ABC - 19.17AB(AB) - 67.22AC(A-C) - 52.46BC(B-C)$$

Where:
A=Modified cassava flour
B=Sago flour
C=Cornstarch

ANOVA test showed that adding modified cassava flour, sago flour, and cornstarch did not significantly affect the texture response of seasoned flour. Adding flour components, A and B separately, as well as the interaction between components AC and BC, increased the crispy texture value, confirmed by a positive constant. Meanwhile, the interaction between components AB and ABC decreased the crispy texture value. The addition of cornstarch had the most significant influence on the increase in crispy texture value, as it had the highest constant value. The type of flour or oil and the seasoned flour ability to absorb and retain water were the main factors affecting the crispness of seasoned flour. The analysis showed that the OHC value of seasoned flour was lower than the WHC value. The higher the WHC, the greater the ability of the material to absorb and retain water. In products like seasoned flour or dough, high WHC allows the product to absorb more water or liquid, increasing the consistency or viscosity of the final product.

The emergence of food aroma was caused by the formation of volatile compounds that easily evaporate. The results of the aroma response measurement ranged from 4.4 to 4.8, confirming that the panelists generally liked the aroma of seasoned flour. Based on the analysis, the aroma response model was a special cubic. The polynomial equation for the aroma response is presented as follows.

$$\text{Aroma: } Y = 3.76A+4.00B+2.90C+3.79AB+5.92AC+4.99BC-15.01ABC$$

Where:
A=Modified cassava flour
B=Sago flour
C=Cornstarch

The addition of modified cassava flour, sago flour, and cornstarch did not significantly affect the aroma response of fried chicken seasoned flour. Adding flour components separately, as well as the interaction between AB, AC, and BC components, increased the aroma value, confirmed by a positive constant. However, the interaction between ABC components decreased the aroma value. The addition of sago flour had the most significant influence on increasing the aroma value because it had the highest constant value. Fried chicken coated with seasoned flour had a distinctive aroma due to the addition of pepper seasoning. The unique properties of pepper include its spicy taste and characteristic aroma. The distinctive aroma also comes from garlic, containing allicin, a chemical compound (Borlinghaus et al., 2021).

The results of the overall response measurement produced values ranging from 4.5 to 5.2, confirming the model to be cubic. The polynomial equation for the overall response is presented as follows.

$$\text{Overall: } Y = 2.76A + 3.49B + 2.29C + 8.20AB + 10.59AC + 7.90BC - 30.02ABC$$

Where:
A=Modified cassava flour
B=Sago flour
C=Cornstarch

Adding modified cassava flour, sago flour, and cornstarch significantly affected the overall response of fried chicken seasoning flour. Adding flour components separately, as well as the interaction between AB, AC, and BC components, increased the overall value, confirmed by a positive constant. However, the interaction between ABC components decreased the overall value. Sago flour had the most significant impact on the overall value increase, followed by modified cassava flour and cornstarch.

Optimization of Seasoned Flour Formula Using D-Optimal Method

The desirability value represents the desired optimization value that can be attained. A desirability index closes to 0 means it will be difficult for the crispy chicken seasoned flour formula to reach the optimal point based on response variables. Meanwhile, a desirability value close to 1 means seasoned flour formula can achieve the optimal formula according to the desired response variables. Using Design Expert program, the optimized components, goal values, limits, and importance at the formula optimization stage are presented in Table 4.

Table 4
Attributes and optimization criteria for seasoned flour formula

Component/ response	Goal	Lower Limit	Upper Limit	Importance
Modified cassava flour	*Maximize*	37.5	42.50	5 (+++++)
Sago flour	*is in range*	27.5	32.50	3 (+++)
Cornstarch	*is in range*	16	20.00	3 (+++)
OHC	*Minimize*	9.8	13.72	5 (+++++)
WHC	*Maximize*	16.67	19.23	5 (+++++)
Moisture content	*Minimize*	7.72	8.18	5 (+++++)
Color	Maximize	4.2	4.70	5 (+++++)
Taste	*Maximize*	4.4	4.90	5 (+++++)
Texture	*Maximize*	3.8	4.80	5 (+++++)
Aroma	*Maximize*	4.4	4.80	5 (+++++)
Overall	*Maximize*	4.5	5.20	5 (+++++)

The optimization stage provided one of the best formula solutions from several suggested formulas, with the highest desirability value of 0.774. The composition of the solution is presented in Table 5. Design Expert program recommended one formula solution with a desirability value closest to 1; hence, the formula could be used for the next test stage.

Table 5
Optimization stage formula solution

Modified cassava flour	Sago flour	Cornstarch	Desirability
41.00	31	16.00	0.774*
40.70	27.6	19.80	0.734
39.50	30.3	18.10	0.583

* The best formula

The best combination comprised 41% modified cassava flour, 31% sago flour, and 16% cornstarch, making up the chosen solution. With a desirability value of 0.774, this formula was expected to meet 77.4% of the optimization target. The formula was anticipated to achieve an organoleptic score of 4.6 for color, 4.9 for taste, 4.6 for texture, 4.8 for scent, and 5.2 for overall quality. It was also expected to have an OHC of 11.3%, WHC of 18.80%, and a moisture content of 7.96%. Figure 4 presents the three-dimensional graph of the optimum formula. In this graph, a low desirability value is represented by the low area, while a high desirability value is shown by the high area.

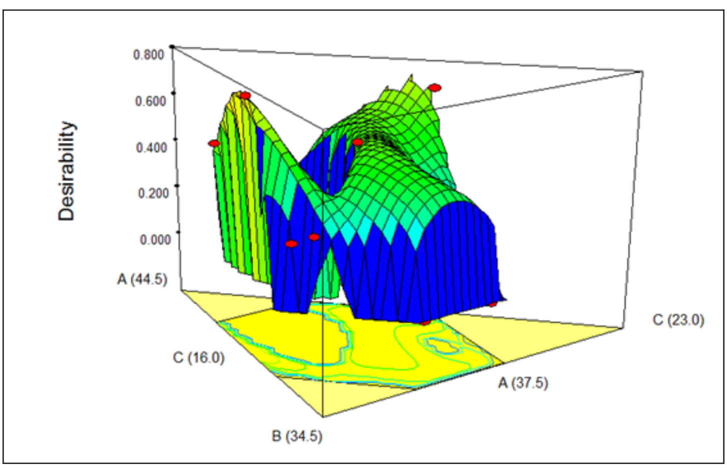


Figure 4. Three-dimensional graph of optimum formula

Verify Optimization Result Formula

The outcomes of the verification step and the predictions made for each response are presented in Table 6. Based on the verification, the data fell in the range predicted by Design Expert program. This was confirmed by the 95% confidence interval (CI) for

WHC, OHC, moisture content, and sensory attributes (color, taste, texture, aroma, and overall acceptance).

Table 6
Results of the verification stages

Response	**Results**		**95% CI low**	**95% CI high**	**95% PI low**	**95% PI high**
	Prediction	**Verification**				
OHC (%)	11.29	12.00	10.00	12.58	8.70	13.88
WHC (%)	18.80	18.52	17.64	19.96	16.92	20.68
Moisture content (%)	7.96	8.06	7.77	8.16	7.57	8.36
Color	4.59	4.6	4.40	4.78	4.31	4.88
Taste	4.92	5.0	4.73	5.12	4.61	5.24
Texture	4.59	4.5	4.08	5.10	3.81	5.37
Aroma	4.83	4.7	4.68	4.98	4.58	5.08
Overall	5.18	5.2	4.99	5.37	4.87	5.49

Characterization of Seasoned Flour Optimal Formula

Moisture, ash, protein, fat, carbohydrate (by difference), and resistant starch content, as well as WHC and OHC obtained from seasoned flour, were analyzed for optimization. Table 7 presents the results of the comprehensive proximate analysis of seasoned flour for crispy fried chicken.

Compared to seasoned flour from Alfani et al. (2019), seasoned flour from this study had a lower water content but higher fat, crude fiber, carbohydrate, and protein content. Seasoned flour from Omeje et al. (2021) had lower crude fiber, ash, and carbohydrate content but higher fat and protein content compared to this study.

Moisture content of seasoned flour met the requirements of the Indonesian National Standards (Badan Standardisasi Nasional, 1998), stipulating a maximum moisture content of 12%. However, the crude fiber content of seasoned flour did not comply with the standards, specifying a maximum crude fiber content of 1.5%. Protein, fat, and carbohydrate content of seasoned flour was not specified in the standards, but the components were suitable for meeting the body's calorie needs. Ash content of seasoned flour did not comply with the standards, also requiring a maximum ash content of 1.5%. The high ash content can be attributed to the addition of seasonings such as salt and baking soda, inorganic salts that remain as ash after ashing. To reduce the high ash content in food ingredients, it can be considered to use soaking methods, using additional ingredients (such as starfruit leaf extract), or choosing types of food ingredients with lower ash content.

The high WHC of seasoned flour allowed the retention of water absorbed during dough preparation. The relatively low OHC of seasoned flour enabled the dough to absorb only a small amount of oil during frying. The resistant starch content in seasoned flour was 5.01±0.01. This component was beneficial for the body, particularly the digestive system. Resistant starch formed a physical barrier that slowed the access of digestive enzymes to starch particles and could reach the large intestine without being altered. As a prebiotic, resistant starch has an advantage over FOS (fructose-oligosaccharides) and inulin because it can bind and retain water in stools, preventing constipation and flatulence, even when consumed in high amounts (Ozturk et al., 2011).

Table 7
Physicochemical and functional composition of optimized seasoned flour

Response	Value (%)
Moisture content	8.06±0.06
Ash content	6.32±0.08
Protein content	2.67±0.03
Fat content	0.40±0.00
Crude fiber content	2.24±0.07
Carbohydrate content	80.31±0.12
Resistant starch	5.01±0.01
Water holding capacity	18.52±0.00
Oil holding capacity	12.00±0.00

Commercial seasoned flour available in the market is made from wheat flour, which lacks high-quality protein due to a deficiency of lysine, and also has low dietary fiber content. In this study, seasoned flour was made without wheat flour, incorporating sago flour and cornstarch to improve nutritional content, particularly resistant starch and fiber content. Therefore, the use of non-wheat seasoned flour was expected to support the local food-based processing industry, as the import of wheat flour was crucial.

CONCLUSION

In conclusion, this study showed that a blend of 41% modified cassava flour, 31% sago flour, and 16% cornstarch produced the best seasoned flour formula, with a desirability value of 0.774. Moreover, the verification results showed that the selected formula had an overall assessment score of 5.2, with individual scores of 4.6 for color, 5.0 for taste, 4.5 for texture, 4.7 for scent, and WHC of 18.52%, along with a moisture content of 8.06%. The composition of the best formula for seasoned flour was 8.06% moisture, 6.32% ash, 2.67% protein, 0.40% fat, 80.31% total carbohydrates, and 5.01% resistant starch. WHC was 18.52%, and OHC was 10.20%. Based on analysis, the use of modified cassava flour, sago flour, and cornstarch produced an acceptable seasoned flour with good physical, chemical, and functional properties. Therefore, the combination of several types of flour into seasoned products was an effective method to increase acceptance and address the problem of malnutrition in the community.

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Hydration Effect and Kinetic Studies for Physical and Hardness Properties of Parboiled Rice MR297

Jhauharotul Muchlisyiyah[1,2], Rosnah Shamsudin[1,3]*, Roseliza Kadir Basha[1], Radhiah Shukri[1] and Syahmeer How[1]

[1]*Department of Process and Food Engineering, Faculty of Engineering, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

[2]*Department of Food Science and Biotechnology, Faculty of Agricultural Technology, Universitas Brawijaya, Jalan Veteran Malang, 65145, Indonesia*

[3]*Laboratory of Halal Science Research, Halal Products Research Institute, Universiti Putra Malaysia, Putra Infoport, 43400 Serdang, Selangor, Malaysia*

[4]*Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

Soaking is a crucial step in the parboiling process that directly impacts the overall quality of parboiled rice. The aim of this study was to investigate the impact of soaking time and temperature on the kinetics of colour, dimensions, and hardness of parboiled rice MR297. In this study, MR297 paddy was soaked at different temperatures (50 °C, 60 °C, and 70 °C) for 1, 2, 3, 4, and 5 hours. Kinetic models assessed for quality changes in parboiled rice included the zero-order, first-order, and second-order models. The data showed that the total colour change (6.95–15.09), chroma (30.46–36.94), browning index (61.59–90.05), length (9.61–10.38 mm), width (2.06–2.37 mm), and thickness (1.80–2.11 mm) increased. Whereas the Hue angle (77.21–74.14) and hardness (3576.61–3167.4) decreased as the temperature (50 °C–70 °C) and time (1–5 hours) of soaking increased. The alterations in colour, dimensions, and hardness were satisfactorily explained by the kinetic models (zero, first, and second-order). Most of the reaction rate constant (k) of the change greatly depended on the soaking temperature. The kinetic models for colour (total colour change, chroma, hue, and browning index), dimensional (length, width, and thickness), and hardness alterations followed the Arrhenius equation. Hence, the fitted kinetic models of the quality alteration can be employed

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E-mail addresses:
lisyah@ub.ac.id (Jhauharotul Muchlisyiyah)
rosnahs@upm.edu.my (Rosnah Shamsudin)
roseliza@upm.edu.my (Roseliza Kadir Basha)
radhiah@upm.edu.my (Radhiah Shukri)
syahmeerhow@gmail.com (Syahmeer How)
*Corresponding author

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to forecast the immersion procedure of parboiled rice MR297. The significance of this study lies in providing predictive models that can optimise the parboiling process for the MR297 rice variety, enabling producers to better control product quality while potentially reducing processing time and energy consumption. These findings contribute to the scientific understanding of rice parboiling and can be valuable for industrial applications in the rice processing industry.

Keywords: Kinetic models, parboiled rice, soaking, quality changes

INTRODUCTION

Rice is a densely nutritious staple food, rich in carbohydrates and containing a small amount of protein, vitamins, fats, and minerals. Parboiled rice has gained widespread popularity around the globe (Balbinoti, Nicolin et al., 2018). Owing to the unique parboiling process, this rice has a distinct texture and flavour from the regular white rice. The parboiling process involves soaking and steaming the rice while it is still enclosed in its husk. This method protects the essential nutrients present in the rice grain (Muchlisyiyah et al., 2023). During the procedure, nutrients are transferred from the rice husk to the rice grains, resulting in greater health benefits than white rice alone (Jayaraman et al., 2019). In addition, the parboiling procedure makes the rice grains harder and less sticky (Onmankhong et al., 2021). The benefits of the parboiled rice include a greater number of nutraceuticals, improved palatability, and increased shelf life (Alexandre, 2020).

The texture is one of the characteristics that distinguishes the parboiled rice from the ordinary white rice, which is known for its special qualities. During the parboiling procedure, the starch in rice grains undergoes structural changes that account for this difference. Parboiling alters the starch structure of rice grains, making them denser and more cohesive (Sittipod & Shi, 2016). Initially, unstructured and loosely bound starch becomes more structured and tightly bound (Li et al., 2021). This results in rice that is softer and chewier after heating. The parboiled rice grains retain their integrity when cooked without becoming excessively sticky or falling apart (Srichamnong & Lasukhang, 2022). Moreover, alterations in the starch structure influence the absorption of water during heating. Rice grains that have been parboiled are better at incorporating water, resulting in rice that is chewier and less sticky (Wiruch et al., 2019). Its delicate, chewy texture, and non-sticky consistency make the parboiled rice the choice for fried rice, congee, and other rice dishes.

The soaking phase before parboiling significantly affects the colour and texture of the parboiled rice produced. During soaking, water permeates the rice grains, altering the starch structure and resulting in firmer and fluffier rice grains after parboiling (Jagtap et al., 2008). Higher temperatures can accelerate the transformation of the starch structure in rice grains during soaking. Rice grains can undergo the desired structural change after the parboiling procedure to gain more resistance to stress during milling and obtain a higher head rice yield (HRY) (Jaiboon et al., 2016). On the other hand, the soaking process will result in a colour change due to the penetration of colour from the husk into the grain (Tian et al., 2014). The soaking process also resulted in some chemical changes, especially in the starch, protein, and lipid substances, which alter both the texture and colour of rice (Fonseca et al., 2011).

Significant research has been conducted on the kinetics and models of the rice parboiling process, especially focussed on the soaking process. The objective of the studies is to understand the physical and chemical changes that occur in rice during the soaking process and to develop a more accurate mathematical model to describe this phenomenon. Previous studies have investigated the hydration kinetics of paddy and rice during soaking (Balbinoti, Jorge et al., 2018; Ji-u & Inprasit, 2019; Nacimento et al., 2022; Sridhar & Manohar, 2003). Martins et al. (2021) conducted a study to investigate the impact of soaking on the moisture content and textural qualities of rice by employing kinetic models (zero, first, and second-order) as well as response surface methods. Rattanamechaiskul et al. (2023) simulated moisture diffusion during soaking paddy and rice and calculated the energy activation of the Khao Dawk Mali 105 paddy Thailand variety. Fonseca et al. (2011) found that temperature and time can optimise the rice soaking process to achieve the highest quality parboiled rice. Other studies have described the multiple steps of parboiling, including soaking, steaming, and drying, using various models (Mahfeli et al., 2022; Oludolapo & Akinoso, 2020; Shaju et al., 2022; Yousaf et al., 2017).

Therefore, modelling the changes in rice as a result of soaking after undergoing the entire parboiling procedure is crucial for predicting and monitoring the quality of parboiled rice, especially in terms of colour, dimensional, and textural changes. In addition to understanding physical changes, this research seeks to develop a mathematical model that can more precisely characterise the soaking process. By employing a valid mathematical model, researchers could better anticipate the degree of parboiling and final characteristics of parboiled rice. However, the alterations in colour and texture caused by soaking are not explained by any established empirical data or formal model. The best-fit model that might be used in research on other agricultural commodities is important as an insight. Kinetic data will make it easier to determine the ideal processing parameters and estimate the quality losses caused by soaking (Devi & Das, 2017; Shamsudin et al., 2021, 2022). The rate equations quantitatively demonstrate the effect for the zero, first, and second orders (Chikpah et al., 2022). Then, the rate equation includes the reaction orders (Azman et al., 2020). The Arrhenius equation was used to explain the parameters under study because they were temperature-dependent. Furthermore, the k revealed that temperature and duration were the two most crucial variables in blanching, which is typically defined by Arrhenius' behaviour. The incorporation of the Arrhenius equation into the kinetic models resulted in the substitution of temperature as a predictor for the quality factors.

This study examines how soaking affects the rate at which the colour, dimensional hardness, and textural hardness of parboiled rice change. The model can also be used to forecast time-dependent variations in the colour, dimensions, and textural firmness of parboiled rice grains resulting from different soaking temperatures and times. Studying the kinetics and models of rice soaking yields useful information for developing efficient parboiling methods. Thus, a comprehensive understanding of parboiled rice soaking conditions and related factors is essential for achieving optimal parboiled rice quality.

METHODOLOGY

Sample Preparation

A local farmer in Tanjung Karang (Selangor, Malaysia) provided fresh MR297 paddy planted in June 2022 and harvested in November 2022. Random samples of fresh paddy grain were collected and afterwards transported to a laboratory setting in sacs for secure conveyance. Upon arrival at the laboratory, the paddy was carefully transferred into polypropylene bags, which were then vacuum-packed. This step was undertaken to further ensure the preservation of the paddy's quality and integrity for subsequent analysis. The paddy was then preserved within a refrigeration unit at 10 °C (Reddy & Chakraverty, 2004). Manual cleaning was performed to remove foreign objects such as sticks, stones, leaves, and other plant components. A moisture content of 19.5% was recorded at the beginning of the experiment.

Parboiling Process

The parboiling method involved three sequential steps: soaking, steaming, and drying. Fresh MR297 paddy was parboiled in a laboratory using a heated soaking procedure. Water (100 mL) in a 250 mL beaker was heated in a water bath (Memmert, WNB22, Germany) to the desired temperatures (50 °C, 60 °C, and 70 °C) (Muchlisyiyah et al., 2024a). Aluminium foil was used to seal the receptacle to prevent excessive evaporation. When the specified temperature (50 °C, 60 °C, and 70 °C) was attained, 100 g of paddy was poured into the water contained in the glass beaker. The rice-to-water ratio was 1:1 (Jannasch & Wang, 2020). A moderate mixing was performed to ensure that all the paddy was soaked. The paddy was immersed for 1, 2, 3, 4, and 5 hours. The rice was then steamed for 20 minutes at 100 °C using a domestic steamer (TEFAL VC1401, France). The rice was cooled at ambient temperature for one hour (Bootkote et al., 2016). This paddy was then dried in an oven at 38 °C for 24 hours (Memmert DO6836, Germany) (Roy et al., 2019; Tian et al., 2018; Villanova et al., 2017). Steaming and drying were not taken into account in this study due to the specific condition of the operation. The paddy was subsequently examined to ascertain its dimensions. The dehulling procedure of desiccated parboiled rice was carried out employing a Satake THU-35 dehusker (THU-35, Satake Corp., Japan), followed by a milling duration of 2.5 minutes using a TM05-C mill (Satake, Japan) (Liang et al., 2008). The milled rice was then analysed for its hardness and colour properties.

Dimensional Measurement

In order to determine the mean size of the paddy grains, a sample of 100 grains was randomly chosen, and the length (L), width (W), and thickness (T) were measured using a Mitutoyo digital vernier calliper with a minimum measurement resolution of 0.01 mm. Figure 1 displays the major (L), medium (W), and minor (T) axis measurements.

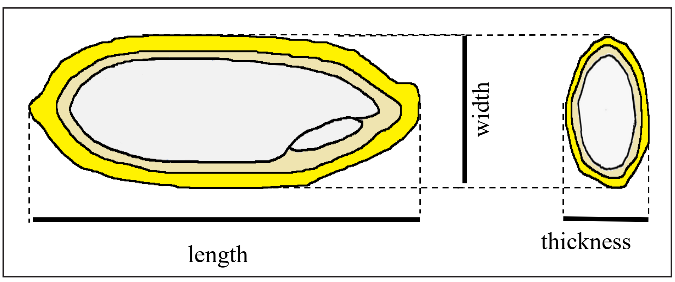

Figure 1. Dimensional characteristic of MR297 paddy (Muchlisyiyah et al., 2024b)

Colour Measurement

Using a colourimeter, 50 g of each milled rice grain variety was selected at random and deposited in a petri dish. The colour properties (CIE L*, a*, and b* scale) of the parboiled rice were identified by placing the colourimeter next to the petri dish containing the samples. The samples were captured using a 40 mm lens (FRU WR10, China) against a white blank paper background. The L*, a*, and b* values can be used to calculate ΔE, browning index (BI), chroma (C*), and hue angle (H) using Equations 1 to 4 (Devi & Das, 2017):

$$\Delta E = [(L_0^* - L^*)^2 + (a_0^* - a^*)^2 + (b_0^* - b^*)^2]^{1/2} \quad [1]$$

$$H = tan^{-1}\frac{b^*}{a^*} \quad [2]$$

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

$$BI = 100\,(x - 0.31)/0.17 \quad [4]$$

Where $x = \frac{a^* + 1.75\,(L^*)}{5.645\,(L^*) + a^* - 3.012\,(b^*)}$

Where L_o, a_o, and b_o are the initial L, a, and b values of the sample, L*, a*, and b* denote the values of L,* a*, and b* under the observed conditions.

Hardness Measurement

Hardness was defined as the maximal force encountered during initial compression. The determination of rice grain hardness was performed using a texture analyser (TA-XT2, Stable Micro Systems, Godalming, UK) by subjecting the grains to pressure. In this study, a stainless-steel probe with a diameter of 5 mm (referred to as P5) was employed to compress individual rice kernels in the direction of their T (minor axis). The compression was conducted at a testing velocity of 2 mm/s, followed by a post-speed of 10 mm/s (Kumar & Prasad, 2018).

Kinetic for Colour, Dimensional, and Hardness Changes

Several published models have described the reaction kinetics-based changes in the dimensions, colour, and hardness of food ingredients as a result of soaking time (Amini et al., 2022; Devi & Das, 2017; Sahoo et al., 2022). Typically, the rate of change can be expressed as Equation 5:

$$\frac{dC}{dt} = -\,kC^n \qquad [5]$$

The equation represents the relationship between the reaction rate constant (k), the concentration of the target parameter (C) at a specific time (t), and the order of the reaction (n). The rate of process change can be assessed using zero-order, first-order, and second-order kinetic models, as outlined in Equations 6 to 8 (Azman et al., 2020).

$$Zero\ order = C = C_0 - kt \qquad [6]$$

$$Fist\ order = Ln\ C = -kt + LnC_0 \qquad [7]$$

$$second\ order = \frac{1}{C} = kt + \frac{1}{C_0} \qquad [8]$$

Where C is the experimental value for each parameter for parboiled rice. C_o is the initial value (in quantitative form).

The temperature dependence of the reaction rate can be described using the Arrhenius equation, which provides insights into the activation energy (*Ea*) involved in the process. Equation 9 represents the Arrhenius equation.

$$K = A\,e^{\frac{Ea}{RT}} \qquad [9]$$

Where A represents the reaction constant at infinite temperature, *Ea* represents the activation energy, R represents the gas constant at 8.3145 J/mol K, and T represents the absolute temperature in Kelvin. The Arrhenius equation is subsequently transformed into Equation 10, where the y-axis represents the Ln k, the x-axis represents the reciprocal of the blanching temperature in Kelvin (1/T), and the slope is determined as the negative ratio of the activation energy to the gas constant (-*Ea*/R).

$$Ln\,K = \frac{Ea}{R}\ \frac{1}{T} + \ Ln\,A \quad [10]$$

Statistical Analysis

The experiment was conducted three times for every test. The statistical analyses were conducted using Minitab 12.0 (PA, USA). The chosen level of significance for assessing differences in analysis of variance (ANOVA) was established at a threshold of $p < 0.05$.

RESULTS AND DISCUSSION

Kinetics of Colour Changes

The changes in the appearance of MR297 rice cultivar samples, soaked at controlled temperatures and after 1, 2, 3, 4, and 5 hours of soaking at 50 °C, 60 °C, and 70 °C soaking temperatures, are presented in Figure 2. After soaking, there is an increasing effect on grain colour as soaking time extends, highlighting the significant impact of soaking duration on

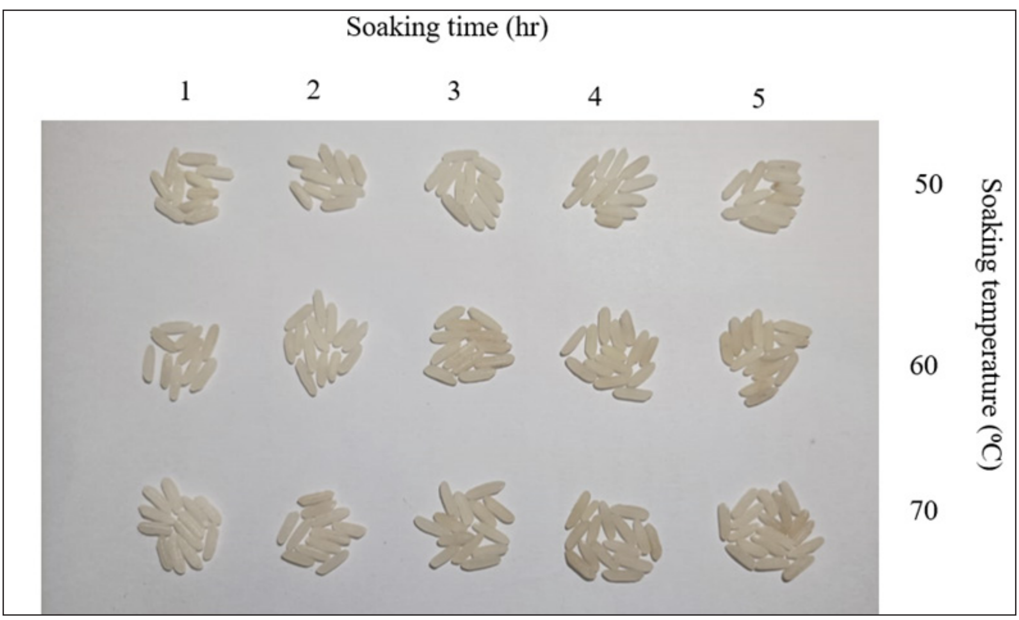

Figure 2. MR297 parboiled rice with different soaking conditions

the physical properties of parboiled rice. The colour properties were obtained using CIE scale variables, specifically a* (representing the presence of redness or greenness), b* (representing the presence of yellowness or blueness), and L* (representing the presence of whiteness or brightness). Afterwards, the L, a*, and b* parameters were used to calculate other colour properties, specifically total ΔE, C*, H, and BI.

Figure 3 depicts the influence of the time and temperature of soaking on many parameters of parboiled rice MR 297, including ΔE*, C*, H, BI, L, W, T, and hardness. The

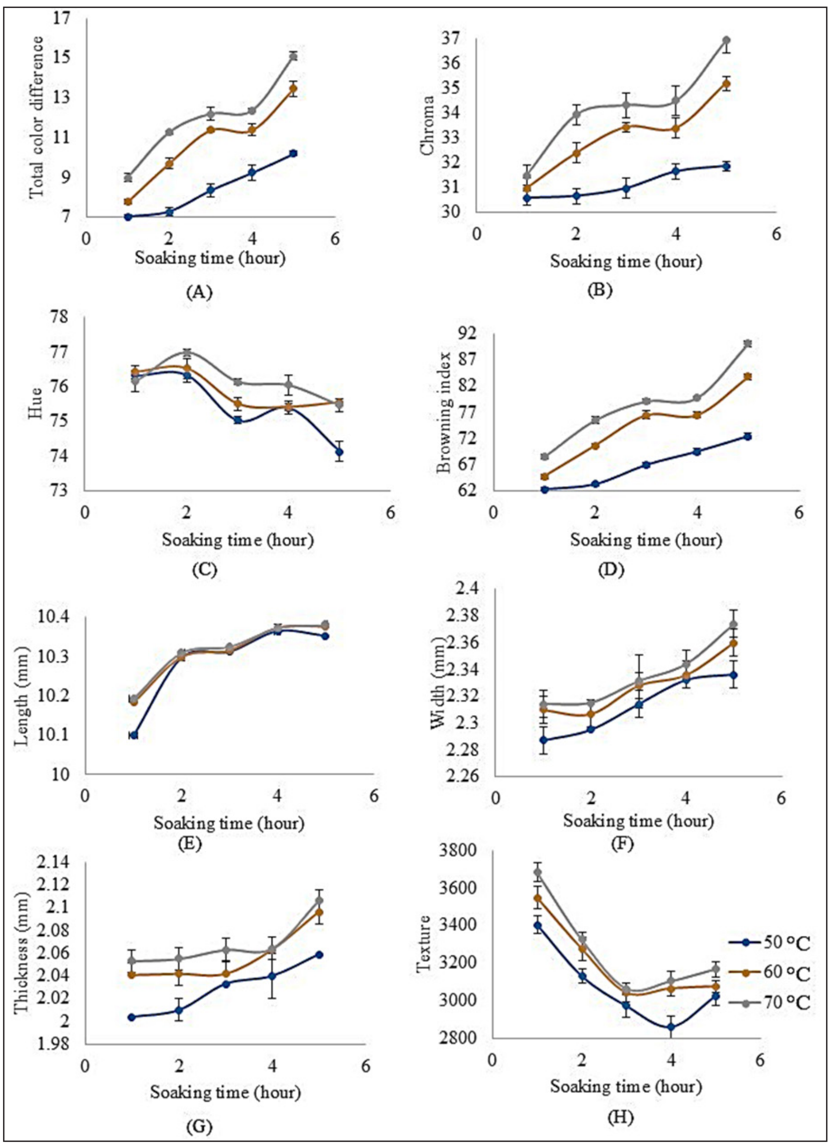

Figure 3. Effect of soaking time and temperature on the (a) total colour change, (b) chroma, (c) hue, (d) browning index, (e) length, (f) width, (g) thickness, and (h) hardness of parboiled rice MR297

immersion method employed in parboiling has the potential to modify the visual appearance of processed rice by affecting its colouration. The application of increased intensity in the parboiling process, characterised by longer soaking duration and higher temperature, resulted in a discernible alteration in the colour of the parboiled rice, manifesting as a yellow-brownish hue. Consequently, this intensified parboiling process led to an overall augmentation in the ΔE, C*, and BI while simultaneously causing a reduction in the H. On the other hand, the longer the time and temperature of soaking, the larger the dimension of the parboiled paddy was observed, thus increasing the L, W, and T. Meanwhile, there was a decrease in the hardness of parboiled rice until 3 hours of soaking, then the hardness increased as the soaking duration increased. Generally, increasing the soaking temperature also increases the hardness. The change in the quality parameters was then plotted in the kinetic models. This study investigates the reaction rate (k) and coefficient of determination (R^2) associated with the zero-order, first-order, and second-order kinetic models in relation to colour parameter changes. Colour changes (ΔE*, C*, H, and BI) as the impact of soaking temperatures (50 °C, 60 °C, and 70 °C) on parboiled rice MR297 are shown in Table 1.

Total Colour Change (ΔE*) is crucial for assessing rice quality, optimising the parboiling process, and predicting outcomes, significantly influencing consumer acceptance and production efficiency. The ΔE notion in parboiled rice, as described by Lamberts, Bie, et al. (2006), denotes the noticeable colour transition from raw rice to the golden or amber tones evident post-parboiling. The transformation is affected by processing parameters like soaking and steaming durations and moisture content (Bootkote et al., 2016; Lv et al., 2009). The transformation is affected by processing parameters like soaking and steaming durations and moisture content. The process of parboiling results in noticeable darkening and yellowing of milled rice, with the extent of colour change contingent upon the intensity of the parboiling application (Lamberts, Brijs, et al., 2006).

ΔE* exhibits a significant decrease during soaking, as evidenced by the high initial values (C = 6.970) and k across all temperature conditions. The magnitude of k increases with increasing temperature, ranging from -0.683 at 50 °C to -1.477 at 70 °C, indicating a faster rate of colour change at higher temperatures. The first and second-order models provide slightly better fits, as indicated by higher R^2 values, but the zero-order model adequately describes the data in most cases. The temperature-dependent colour shift aligns with observations in other food items, like pumpkins and popped rice, where elevated temperatures result in more significant colour changes (Chikpah et al., 2022; Devi & Das, 2017). The research conducted by Oli et al. (2016) corroborates this, indicating that ΔE values increase when soaking temperatures surpass 90 °C, highlighting the essential influence of temperature in the parboiling procedure.

Chroma (C*) is an essential aspect of colour perception, denotes the intensity or saturation of colour and is one of three primary properties with hue (H) and lightness (L*). In the specific domain of parboiled rice, the term "chroma change" applies to the

Table 1
The reaction rate (k) and coefficient of determination of the zero, first, and second-order kinetic models of changes of dimensions of the parboiled paddy and colour parameter (ΔE, chroma, hue, and browning index) and hardness of parboiled rice MR297 Long grain rice due to the soaking step in parboiling

Colour change parameter	**Temperature (°C)**	**Zero-order**				**First Order**				**Second Order**			
		C_0	**k**	**R^2**	**SEE**	**C_0**	**k**	**R^2**	**SEE**	**C_0**	**k**	**R^2**	**SEE**
ΔE* (total colour change)	50	6.970	-0.683	0.919	0.425	1.942	-0.082	0.930	0.047	0.144	-0.010	0.939	0.005
	60	6.970	-1.283	0.964	0.516	1.942	-0.131	0.957	0.058	0.144	-0.014	0.936	0.008
	70	6.970	-1.477	0.948	0.725	1.942	-0.140	0.922	0.086	0.144	-0.014	0.871	0.012
Chroma	50	30.455	-0.302	0.906	0.204	3.416	-0.010	0.908	0.007	0.033	-0.000	0.910	0.000
	60	30.455	-0.915	0.954	0.421	3.416	-0.028	0.956	0.013	0.033	-0.001	0.956	0.000
	70	30.455	-1.196	0.928	0.699	3.416	-0.036	0.927	0.021	0.033	-0.001	0.925	0.001
Hue	50	77.214	0.556	0.893	0.402	4.347	0.007	0.893	0.005	0.013	9.72E-05	0.892	7.08E-05
	60	77.214	0.353	0.822	0.343	4.347	0.005	0.822	0.005	0.013	6.07E-05	0.822	5.90E-05
	70	77.214	0.284	0.669	0.417	4.347	0.004	0.670	0.006	0.013	4.87E-05	0.671	7.14E-05
Browning Index	50	61.699	-2.257	0.945	1.136	4.122	-0.034	0.950	0.016	0.016	-5.12E-04	0.954	2.34E-04
	60	61.699	-4.318	0.967	1.666	4.122	-0.060	0.967	0.023	0.016	-8.43E-04	0.963	3.46E-04
	70	61.699	-5.118	0.951	2.422	4.122	-0.068	0.948	0.034	0.016	-9.23E-04	0.935	5.06E-04
L (length)	50	10.096	-0.060	0.822	0.058	2.312	-0.006	0.821	0.006	0.099	-0.001	0.820	0.001
	60	10.096	-0.057	0.903	0.039	2.312	-0.006	0.901	0.004	0.099	-0.001	0.899	0.004
	70	10.096	-0.057	0.886	0.043	2.312	-0.006	0.884	0.004	0.099	-0.001	0.882	0.000
W (Width)	50	2.284	-0.012	0.949	0.006	0.826	-0.005	0.950	0.003	0.438	-0.002	0.950	0.001
	60	2.284	-0.014	0.942	0.007	0.826	-0.006	0.942	0.001	0.438	-0.003	0.942	0.001
	70	2.284	-0.016	0.944	0.008	0.826	-0.007	0.944	0.004	0.438	-0.003	0.945	0.002
T (Thickness)	50	2.000	-0.012	0.946	0.006	0.693	-0.006	0.947	0.003	0.500	-0.003	0.948	0.002
	60	2.000	-0.016	0.861	0.013	0.693	-0.008	0.862	0.006	0.500	-0.004	0.861	0.003
	70	2.000	-0.016	0.810	0.017	0.693	-0.008	0.808	0.008	0.500	-0.004	0.806	0.004
	50	3581.630	131.329	0.785	143.618	8.184	0.041	0.782	0.045	2.792E-04	1.27E-05	0.777	1.42E-05
Hardness	60	3581.630	120.762	0.829	114.787	8.184	0.037	0.829	0.035	2.792E-04	1.11E-05	0.828	1.06E-05
	70	3581.630	116.446	0.704	157.803	8.184	0.035	0.704	0.047	2.792E-04	1.03E-05	0.702	1.40E-05

alteration in the intensity or saturation of colour exhibited by the rice because of the parboiling procedure (Bhattacharya, 1996). In parboiled rice, chromatic alterations signify the movement of pigment from the outer to inner layers during the parboiling process, influencing the colour intensity and saturation (Lamberts, Brijs et al., 2006; Lamberts, Bie et al., 2006). The chroma values (C*) follow a similar trend to ΔE*, with high initial values (C_0 = 30.455) that decrease during soaking. The temperature dependence of k is more pronounced for chroma, with k at 70 °C being nearly four times higher than at 50 °C. The first- and second-order models yield lower C_0 and k values but comparable R^2 values to the zero-order model. This pattern parallels observations in other culinary processes, such as popped rice and pumpkins, where the application of heightened heat leads to more pronounced colour changes (Chikpah et al., 2022; Devi & Das, 2017).

The hue angle (H) indicates visual colour perception and is an essential measure for evaluating the quality of parboiled rice (Bett-Garber et al., 2012). In contrast to the other colour parameters, hue exhibits a slight increase during soaking, as indicated by the positive k values. However, the magnitude of change in hue is relatively small compared to the other parameters, and the k decreases with increasing temperature. The reaction order has a minimal impact on the kinetic description of hue, with all three models providing similar C_0, k, and R^2 values. These findings correspond with the extensive food science literature, where analogous thermal processes influence colour characteristics, highlighting the significance of temperature and processing conditions in colour dynamics (Dixon et al., 2020).

The browning index (BI) quantitatively assesses the degree of browning or colour change in rice after parboiling, principally resulting from browning processes and pigment degradation (Lamberts, Brijs et al., 2006). The parboiling process involves Maillard reactions, as seen by furosine levels, which are prominent in the outer bran layers and the endosperm, signifying the progression of browning (Taghinezhad et al., 2015). The BI successfully reflects these changes, utilising kinetic models for comprehensive quantitative analysis. The browning index undergoes a substantial decrease during soaking, with high initial values (C_0 = 61.699) and large negative k values. The effect of temperature on the browning index is significant, with the k at 70 °C being more than double that at 50 °C. The first and second-order models offer marginal improvements in R^2 compared to the zero-order model. The temperature dependency is essential for comprehending the BI dynamics since elevated temperatures expedite pigment degradation and Maillard reactions, resulting in more significant colour alterations. This conclusion aligns with research on other food products, indicating that elevated temperatures augment browning indices (Devi & Das, 2017). Moreover, initial hydration can alleviate certain browning effects during milling; however, extended hydration causes pigments to penetrate further into the endosperm, rendering them more difficult to eliminate (Oli et al., 2016).

The length of rice grains is a key quality attribute affecting consumer preference and market value. The initial length of the paddy (C_0) is 10.096 mm, which decreases slightly during soaking as indicated by the negative reaction k. The magnitude of k is consistent across all temperature conditions, ranging from -0.057 to -0.060, suggesting that the change in length is not significantly influenced by temperature. The first- and second-order models provide similar fits to the data as the zero-order model, with comparable R^2 values.

The width of rice grains affects the texture and mouthfeel of the cooked rice. The width of the paddy follows a similar trend to length, with an initial value (C_0) of 2.284 mm that decreases during soaking. The k are negative and increase in magnitude with increasing temperature, indicating a faster rate of change at higher temperatures. The first and second-order models yield similar k and R^2 values to the zero-order model, suggesting that the reaction order has minimal impact on the kinetic description of width change.

The thickness of rice grains is critical for ensuring uniform cooking and texture. The initial thickness of the paddy (C_0) is 2.000 mm, which decreases during soaking as evidenced by the negative k values. The k values are consistent across the temperature range of 50 °C to 70 °C, indicating that temperature has a limited influence on the rate of thickness change. The R^2 values for the zero, first, and second-order models are comparable, with a slight decrease at higher temperatures. The textural characteristics of parboiled rice MR297, namely hardness, were meticulously measured utilising zero, first, and second-order kinetic models to analyse the influence of soaking temperature on textural alterations in rice (Table 1). The hardness of the rice exhibits a significant decrease during soaking, with high initial values (C_0 = 3581.630) and large positive k. The magnitude of k decreases with increasing temperature, suggesting that higher temperatures lead to a faster rate of softening. The R^2 values for the zero, first, and second-order models are similar, indicating that the reaction order has minimal impact on the kinetic description of hardness change. The decreasing effectiveness of hardness reduction at elevated temperatures may result from the denaturation of proteins or other thermally stable constituents inside the rice grains.

The identified trends among the models and temperatures highlight the intricate relationship between temperature, soaking duration, and the textural characteristics of rice. The decline in k across all models with rising temperature indicates a gradual stabilisation or restriction in textural alteration, emphasising the thermal sensitivity of rice hardness during the parboiling process. The outcomes of this study enhance the comprehension of optimal processing parameters necessary for attaining specific textural qualities in parboiled rice, with ramifications for industrial practices and product quality assurance.

Energy (Ea) Analysis

Table 2 presents the activation energy (Ea) and R^2 values for zero-, first-, and second-order kinetic models, assessing the influence of soaking on the physical and textural attributes of parboiled rice MR297 (Mohapatra & Bal, 2015). These models help understand the energy required for changes in attributes like colour (ΔE*, Chroma, Hue), texture (Hardness), and dimensions (Length, Width, Thickness), which are crucial for optimising the soaking process to maintain rice quality essential for consumer satisfaction and market competitiveness.

Table 2
The activation energy (Ea) and coefficient of determination (R^2) of the kinetic models (zero, first, and third order) according to the changes of parameters of MR297 parboiled paddy and milled rice due to soaking

Parameters	**Zero Order**		**First Order**		**Second Order**	
	***Ea* (kJ/mol)**	**R^2**	***Ea* (kJ/mol)**	**R^2**	***Ea* (kJ/mol)**	**R^2**
ΔE*	35.781	0.892	24.784	0.852	15.376	0.768
C*	63.762	0.900	60.413	0.894	57.200	0.889
Hue	-31.138	0.966	-31.555	0.966	-31.922	0.965
Browning Index	37.942	0.908	32.507	0.893	27.321	0.874
Length	-2.669	0.787	-2.764	0.789	-2.886	0.791
Width	13.676	1.000	13.297	1.000	12.925	1.000
Thickness	13.311	0.857	12.718	0.856	12.158	0.854
Hardness	-5.565	0.958	-7.562	0.970	-9.613	0.976

For ΔE*, Ea decreases from 35.781 kJ/mol in the zero-order model to 15.376 kJ/mol in the second-order model, indicating that less energy is needed for colour changes as model complexity increases (R^2 of 0.892). Chroma, sensitive to soaking conditions, shows Ea ranging from 63.762 to 57.200 kJ/mol, necessitating precise control to preserve colour quality. Hue demonstrates negative Ea across all models, suggesting that colour changes during soaking may lead to energy release, potentially due to pigment breakdown, with first-order kinetics providing a reliable prediction of these changes. The browning index varies from 37.942 to 27.321 kJ/mol, with first-order kinetics offering a more precise description of browning reactions.

Dimensional changes show varied responses; length displays a slight negative Ea, indicating low resistance to alterations during soaking, while width shows a notable Ea of 13.676 kJ/mol, reflecting its high sensitivity to soaking conditions with exceptional R^2 values of 1.000. Thickness Ea, ranging from 13.311 to 12.158 kJ/mol, suggests that these models intensively represent the fluctuations in thickness during soaking. The textural properties indicate that the hardness of rice grains decreases upon soaking, with the second-order model showing the lowest Ea value at -10.192 kJ/mol, illustrating the grains' natural propensity to soften —a key factor for achieving optimal culinary quality. This analysis underscores the need for meticulous regulation of soaking parameters to maintain optimal colour characteristics in parboiled rice, as significant energy is required for colour alterations.

CONCLUSION

The current study examined the impact of soaking temperature and time on the kinetics of colour, size, and hardness alteration. The quality change of parboiled rice MR297 was comprehensively characterised by the kinetic models (zero, first, and second-order). ΔE*, C*, BI, L, W, and T increased, whereas H and hardness decreased throughout the soaking process. The best fit for the colour parameter was the browning index following the first-order reaction kinetic model ($R^2 = 0.967$). For the dimensions, the best-plotted parameter for the kinetic model was the W in the second-order reaction kinetic model ($R^2 = 0.950$). The rate of change of colour (ΔE*, C*, H, and BI), dimensional (L and T), and hardness are significantly affected by the soaking temperature and follow the Arrhenius equation. Hence, the colour, dimensional, and hardness change kinetics parameters can be valuable in forecasting alterations in the soaking quality of parboiled rice MR297 during the hot water soaking process.

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Foliar Sprays of Biostimulants and PGRs Improve the Reproductive Parameters of Cacao Under Water Stress

Vences Cuyno Valleser[1]* and Calixto Mabesa Protacio[2]

[1]*Department of Horticulture, College of Agriculture, Central Mindanao University, University Town Musuan, 8710 Bukidnon, Philippines*
[2]*Institute of Crop Science, College of Agriculture and Food Science, University of the Philippines Los Baños, 4031 Laguna, Philippines*

ABSTRACT

One of the essential factors influencing yield of crops is the availability of water. This study was conducted to determine the efficacy of biostimulants (oligocarrageenan and oligochitosan) and plant growth regulators (PGRs) such as cytokinin and paclobutrazol applied as foliar spray on the pod retention and yield of six-year-old grafted 'UF 18' cacao trees under a monocropped system during the dry season in Type III climate of the Philippines. Tap water (control), oligocarrageenan (150 ppm), oligochitosan (150 ppm), cytokinin (40 ppm), and paclobutrazol (500 ppm) were applied as a foliar spray onto cacao trees with young cherelles (BBCH 70-72) under irrigated and rainfed conditions. Among the foliar treatments, oligochitosan caused a higher flowering intensity after four months. Paclobutrazol reduced the incidence of cherelle wilt, while cytokinin improved the pod retention of cacao. The biostimulants and PGRs generally enhanced the leaf chlorophyll content of cacao, increased the mean seed fresh weight, produced higher percentages of full beans and lesser flat beans per tree, and had a better bean count than the control. The cacao trees treated with oligocarrageenan produced pods with heavier dry beans. Moreover, heavier dry bean weight per tree were recorded from cytokinin-, PBZ-, and oligocarrageenan-treated trees. Foliar sprays of biostimulants and PGRs resulted in a significant increase in yield per tree of cacao under water stress.

Keywords: Cytokinin, oligocarrageenan, oligochitosan, paclobutrazol, plant activators

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E-mail addresses:
vcvalleser@gmail.com (Vences Cuyno Valleser)
cmprotacio1@up.edu.ph (Calixto Mabesa Protacio)
*Corresponding author

INTRODUCTION

Cacao (*Theobroma cacao* L.) is one of the high-value crops cultivated in the Philippines but local production ranges only from 0.5 to 1.0 kg per tree per year or equal to about 500 to 1000 kg per hectare

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(Department of Agriculture, 2017). The target yield per tree per year of cacao dried beans is set at 2.0 kg according to the 2016-2022 Philippine Cacao Roadmap (DA, 2017). However, it cannot be denied that the productivity of a cacao tree is limited by the prevailing environmental conditions.

Wood and Lass (2001) identified rainfall as the most important environmental factor impacting cocoa production. Water limitation or drought stress could have negative effects on growth, flowering, pod setting, pod retention (Adjaloo et al., 2012; Matias et al., 2024), and yield of cacao trees. Long dry spells with less than 100 mm of rain per month for more than three months can significantly impair cacao tree growth and yield (Lahive et al., 2019). Therefore, cacao production is favourable in regions where rainfall is evenly distributed throughout the year and have no dry season. According to Coronas (1920) classification, this is the Type IV climate of the Philippines. In regions with no distinct maximum rain periods and with one to three months of dry season which is the Type III climate of the country (Coronas, 1920), a higher yield of cacao could be attained if supplemental irrigation during the dry months is provided.

Water-limiting conditions during the dry season in Type III climate can be mitigated through the application of biostimulants and plant growth regulators (PGRs). Biostimulants are recognised in crop production since these substances enhance mineral nutrition uptake, provide protection, and improve the yield and quality of plants under abiotic conditions (Franzoni et al., 2022). In addition, biostimulant application is a sustainable agricultural practice to maintain crop yield under reduced fertiliser situations (Gupta, 2020). Biostimulants are categorised as microbial and non-microbial. Chitosan and seaweed extract (i.e., carrageenan) are examples of non-microbial biostimulants (Li et al., 2022), of great interest in the global crop industry, which are known to stimulate growth and provide protection to various crop species. Chitosan and carrageenan are long-chain carbohydrates derived from crustacean and fungal exoskeletons, and seaweed (algae) origins respectively. Irradiation of chitosan and carrageenan will result in the formation of shorter chain-oligosaccharides, and when they are bioactive, these are called oligosaccharins (oligochitosan and oligocarrageenan). Oligosaccharins act as a signal molecule that could activate several plant physiological processes leading to growth stimulation and plant defense (Albersheim & Darvill, 1985).

Plant growth regulators (PGRs) are organic compounds that could regulate the growth and development of plants. Paclobutrazol (PBZ) is one of the known plant growth regulators that enhances accumulation of carbohydrates and chlorophyll synthesis in plant leaves which supports the reproductive structures of cacao trees. On the other hand, cytokinin is a hormone that prevents chlorophyll degradation (Taiz & Zeiger, 2010), thus increasing crop yield even under abiotic stress condition.

Despite the growth regulatory effects of biostimulants and PGRs on several crops, there is no existing information on their effects on the pod retention and yield of cacao trees during water-limiting conditions.

As the cacao pods are developing, proteins and photoassimilates are being used up. In this study, it is hypothesised that foliar spraying of biostimulants and PGRs will influence cacao physiology, thereby enhancing pod retention and yield of tree during water-limiting conditions.

Hence, this study was conducted to determine the effects of foliar applications of biostimulants and PGRs on the pod retention and yield per tree of cacao during the dry season in Type III climate.

MATERIALS AND METHODS

This study was conducted from December 2022 to June 2023 in a six-year-old monocrop 'UF 18' cacao plantation in Bangcud, Malaybalay City, Bukidnon, Philippines (Latitude 7.991817°, Longitude 125.131607°, at 326.71m of altitude). Cacao trees were planted at a spacing of 3x3 metres.

The experiment site belongs to the Type III climate where there is no distinct maximum rain period, and with a short dry season (Coronas, 1920) within a year. Weather data such as temperature and rainfall (Figure 1) were obtained from the weatherlink.com through the Mt. Kitanglad Agricultural Development Corporation situated in Lurugan, Valencia City, Bukidnon.

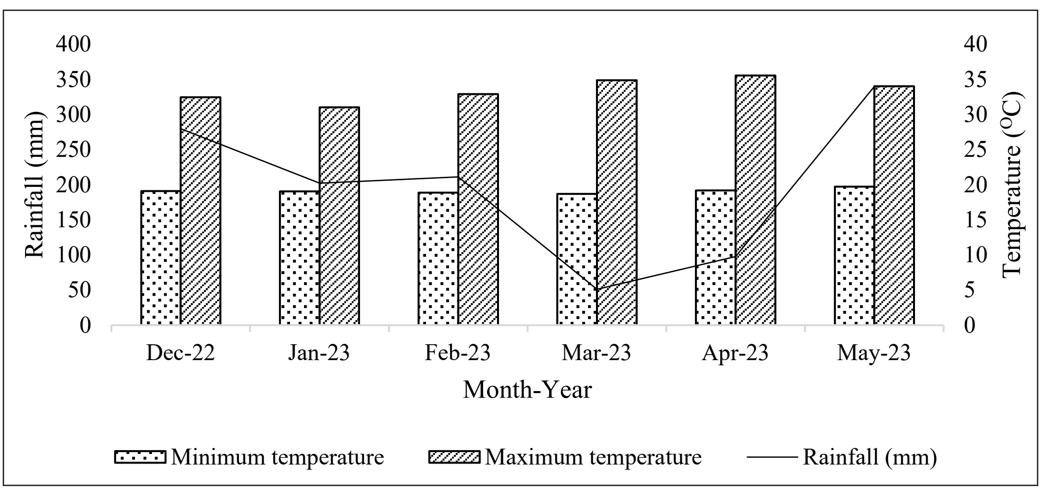

Figure 1. Minimum and maximum temperatures and amount of rainfall data

The soil bulk density of the area is 0.9 g/cm^3, 1.219 g/cm^3, and 1.030 g/cm^3 at depths of 0-30 cm, 30-60 cm, and 60-90 cm respectively. The data imply that the soil is favourable for root growth as well as tree growth of cacao. As a basic principle, the soil becomes compacted and unfavourable for plant growth if the soil bulk density is ≥2 g/cm^3.

To measure soil moisture, probes (gypsum blocks) were installed below the soil within the depths of 0.3 m, 0.6 m, and 0.9 m in both experiment set-ups (with irrigation and without irrigation). Soil moisture was measured at weekly intervals using a Delmhorst soil moisture tester (Figure 2). Weekly soil moisture data is expressed in centibars (Figure 3) using the conversion table specified in the DelmhorstTM KD-1 manual (http://www.moisturemetersdelmhorst.com/content/delmhorst/manual/KS-D1-Soil-moisture-meter-Manual-rev-04-11.pdf).

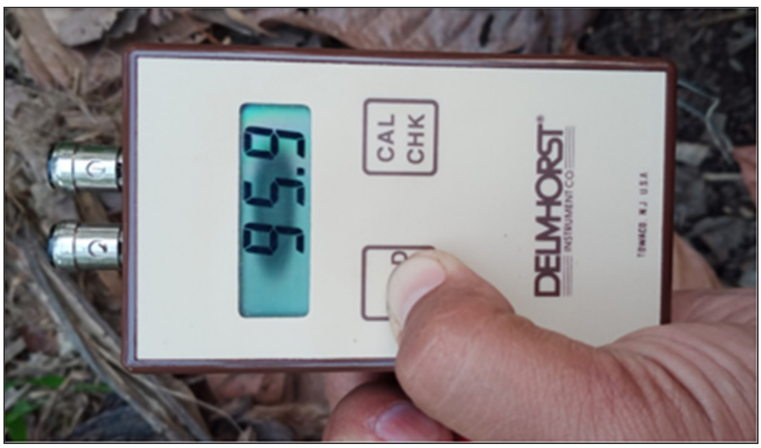

Figure 2. Measuring soil moisture using the Delmhorst™ KS-D1 (Towaco, N.J. USA) soil moisture metre

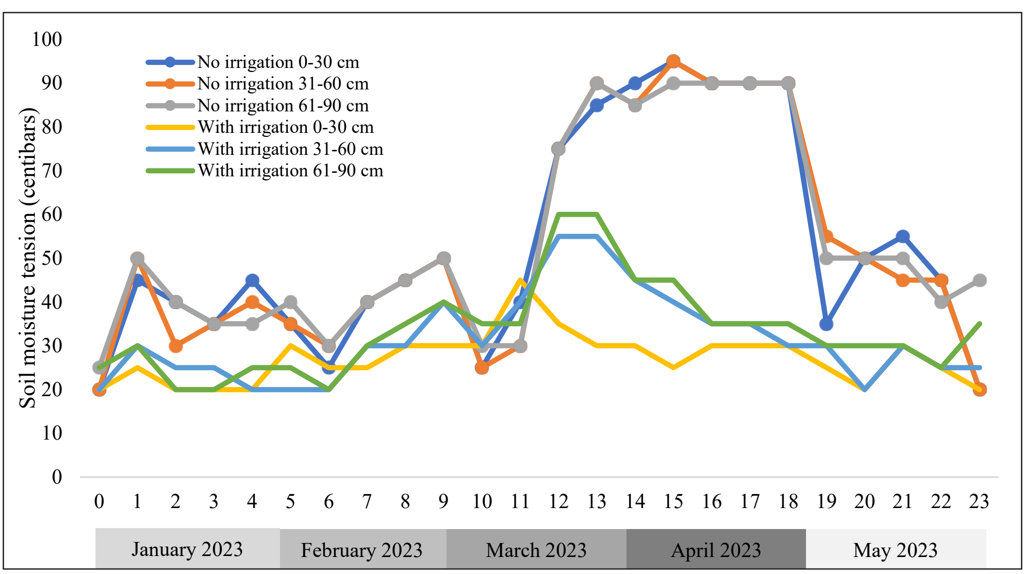

Figure 3. Soil moisture tension in experiments with irrigation and without irrigation

Six-year-old ‘UF 18’ cacao with uniform growth and cherelles ([BBCH 70-72] Azogue et al., 2023) set were selected as experimental trees. This study comprised two sets of experiments. Cacao trees in the first experiment set-up were not provided with irrigation throughout the experiment period. In the second experimental set-up, cacao trees were supplemented with irrigation based on the farmers’ practice (5 li/tree applied twice a week or at least once a week in the absence of rainfall). Each experiment set-up was arranged in a randomised complete block design (RCBD) with three replications. There were 15 experimental units per set-up.

Treatments consisted of 1) Tap water (negative control); 2) 150 ppm oligocarrageenan; 3) 150 ppm oligochitosan; 4) 40 ppm cytokinin; and 5) 500 ppm paclobutrazol. The source of oligocarrageenan, oligochitosan, cytokinin, and PBZ was VitalGro carrageenan® (5,500 ppm carrageenan byproducts as active ingredient), Chitosan oligosaccharide® soluble powder (99% a.i.), Stoller X-Cyte® (0.04% cytokinin as kinetin), and Greenfast® (25SC PBZ) respectively. Treatments were sprayed using a hand-operated shoulder sprayer (Lotus LTGT5000PSX) with an operating pressure of 0.3Mpa. The spraying volume was 5 L tree^{-1}. The pH of the water used to prepare the solutions was 7.1. Above ground parts of the trees including cherelles (BBCH 70-72) were sprayed with the treatment solution. These treatments were applied to plants in both sets of experiments (irrigated and rainfed).

Data Gathered

Leaf Chlorophyll Index

The leaf chlorophyll index was measured using the FieldScout CM 1000 (Spectrum Technologies Inc, 360 Thayer Court, Aurora, IL 60,504). Chlorophyll index was measured from the youngest fully-expanded sun leaf and shade leaf from four branches of each experimental tree. Shade leaves are those with larger leaf area and thinner. On the other hand, sun leaves have smaller leaf area and thicker (Taiz & Zeiger, 2010).

Cushions and Flowers

Cushions are former leaf axils where flowers are borne. Cushions and flowers were counted and recorded before treatment application. Subsequent counting and recording of these parameters per tree were done at monthly intervals.

Cherelle Wilt

The initial count of healthy cherelles (BBCH 70-72 stages) from each experimental tree was also counted and recorded. Cherelles (BBCH 70, BBCH 71, and BBCH 72) developed, after treatment application, were monitored and recorded at weekly intervals. Cherelle wilt incidence was monitored and recorded at weekly intervals. Existing cherelles (BBCH 70-72) before treatment application and those that developed after treatment application until 15 weeks were monitored until they reached the BBCH 75 (end of the cherelle wilt phase). Cherelle wilt incidence was then obtained using the formula below:

$$\text{Cherelle wilt (\%)} = \frac{\text{Number of cherelles wilted}}{\text{Number of cherelles developed}} \times 100$$

Pod Retention

Pod development was monitored (Figure 4) at weekly intervals until maturity and ripening. The total number of harvested cacao pods was counted manually. Pod retention per tree was obtained using the formula below:

$$\text{Pod retention (\%)} = \frac{\text{Number of pods harvested}}{\text{Initial number of cherelles set}} \times 100$$

Pod Weight

Cacao pods were harvested at the BBCH 81 stage. Individual pods harvested were weighed using a pre-calibrated weighing scale. The mean pod weight was computed using the formula:

$$\text{Pod weight (g)} = \frac{\text{Pod weight (g)}}{\text{Number of pods harvested}}$$

Fresh Weight of Seeds per Pod

Fresh seeds from harvested pods were collected and weighed. This parameter was computed using the formula:

$$\text{Fresh weight of seeds per pod (g)} = \frac{\text{Weight (g) of fresh seeds collected}}{\text{Number of pods harvested}}$$

Figure 4. Reproductive development of 'UF 18' cacao applied with different foliar treatments under irrigated and rainfed conditions (photos taken at different dates)

Number of Seeds per Pod

Fresh seeds from harvested pods were collected. This parameter was computed using the formula:

$$\frac{\text{Number of fresh seeds collected=Number of seeds per pod}}{\text{Number of pods harvested}}$$

Seed Fresh Weight

Fresh seeds from harvested pods were collected. This parameter was computed using the formula:

$$\text{Fresh weight per seed (g)} = \frac{\text{Weight of fresh seeds collected}}{\text{Number of seeds collected}}$$

Number of Full Beans per Tree

Seeds from harvested pods were collected and dried to 7% moisture. The total number of full beans collected from each tree was then recorded.

Weight of Full Beans per Tree

Seeds from harvested pods were collected and dried to 7% moisture. The weight of full beans collected from each tree was then recorded.

Number of Flat Beans per Tree

Flat beans are those seeds with cotyledons that are too thin (Wood & Lass, 2001). Seeds from harvested pods were collected and dried to 7% moisture. The total number of flat beans collected from each tree was then recorded.

Weight of Full and Flat Beans per Tree

Seeds from harvested pods were collected and dried to 7% moisture. The weight of flat beans collected from each tree was then recorded.

Full Beans per Pod

Full beans from harvested pods were collected. This parameter was expressed in percentage using the formula:

$$\text{Full beans per pod (\%)} = \frac{\text{Number or weight of full beans}}{\text{Full beans + flat beans}} \times 100$$

Flat Beans per Pod

Beans from healthy pods were collected. This parameter was expressed in percentage using the formula:

$$\text{Flat beans per pod (\%)} = \frac{\text{Number or weight of flat beans}}{\text{Full beans + flat beans}} \times 100$$

Dried Bean Weight

Dried beans were weighed and average dried bean weight was computed using the equation:

$$\text{Dried bean weight (g)} = \frac{\text{Weight of dried beans}}{\text{Number of dried beans}}$$

Bean Count

Bean count is the total number of cacao beans required per 100 grammes. Using the formula below, the bean count was computed:

$$\text{Bean count } (n\text{BEAN}) = \frac{n\text{WHOLE} \times 100}{m\text{WHOLE}}$$

Where nWHOLE is the number of whole beans, and mWHOLE is the mass of whole beans (g).

Dry Weight (DW) per Tree of Marketable Beans

The equation below was used to compute the DW for tree of marketable beans as influenced by the foliar treatments:

DW per tree of marketable beans = no. of pods retained × no. of full beans/pod × DW of full beans

Estimated Yield per Tree

The cherelles that reached BBCH 75 (Azogue et al., 2023) were considered in the computation of this parameter. The number of BBCH 75 pods that developed in the irrigated trees was considered as the pods retained for the wet season. On the other hand, the BBCH 75 pods that developed in the trees in the rainfed experiment was considered as the pods retained for the dry season. The following formula was used to compute for the:

Estimated yield per tree (dry season)	=	no. of pods retained (rainfed experiment) × no. of full beans/pod × DW of full beans;
Estimated yield per tree (wet season)	=	no. of pods retained (irrigated experiment) × no. of full beans/pod × DW of full beans; and
Estimated yield per tree per year	=	Yield per tree estimate (dry season) + Yield per tree estimate (wet season)

Statistical Analysis

Individual analyses of variance in randomised complete block design (RCBD) for each parameter was performed using the Statistical Analysis for Agricultural Research (STAR 2.0.1) software (http://bbi.irri.org/products). To examine the interaction between irrigation practice and foliar treatments for each parameter, combined analysis of variance was performed for parameter(s) in the individual analyses of variance that satisfies the assumptions of ANOVA for homogeneity of means. Significant differences between treatment means were separated using the Tukey's Honest Significant Difference (HSD) test at 0.05 level.

RESULTS

Effects of Biostimulants and PGRs on the Chlorophyll Index of Cacao Leaf Under Different Irrigation Practices

Cacao Sun Leaves

There was no significant interaction observed between irrigation practice and foliar treatment on the chlorophyll reading of cacao sun leaves from 1MAT to 4MAT. Significant effects of foliar treatments on the cacao sun leaf chlorophyll reading at 2MAT and 3MAT were observed only in the rainfed experiment (Figure 5) and not in the irrigated experiment. At 2MAT, the foliar spray of oligocarrageenan, and PBZ treatments resulted in higher chlorophyll index in cacao sun leaves compared to the control. At 3MAT, the chlorophyll index of the cacao sun leaves from trees applied with cytokinin and paclobutrazol was higher compared to other foliar treatments (Figure 5).

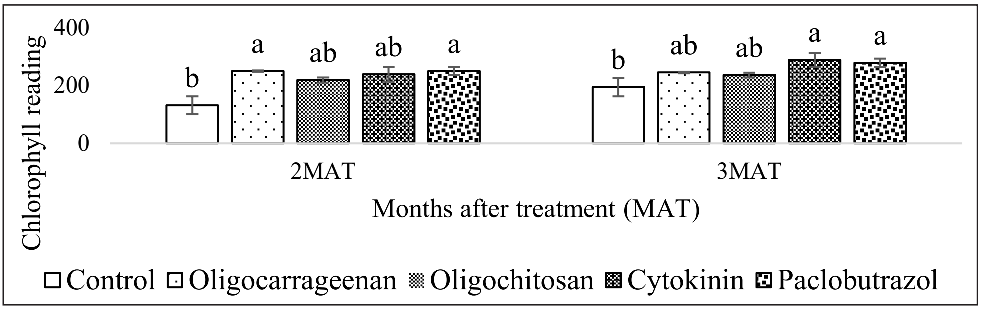

Figure 5. Effects of foliar treatments on the chlorophyll index of cacao sun leaves as measured by the FieldScout CM 1000 at 2MAT and 3MAT under rainfed condition
cv (%)= 27.43; p= .0272* (2MAT)
cv (%)= 18.52; p= .0450* (3MAT)
Treatment means with the same superscript within 2MAT and 3MAT respectively are not significant according to Tukey's HSD test (p ≤.05)

Cacao Shade Leaves

Significant interaction between irrigation practice and foliar treatment on the chlorophyll reading of cacao shade leaves from 1MAT to 4MAT was not observed. Significant effects of foliar treatments on shade leaf chlorophyll reading at 3MAT was observed only in the irrigated experiment (Figure 6). The chlorophyll index of the cacao shade leaves at 3MAT was higher in trees applied with biostimulants and PGRs treatments compared to the control (Figure 6).

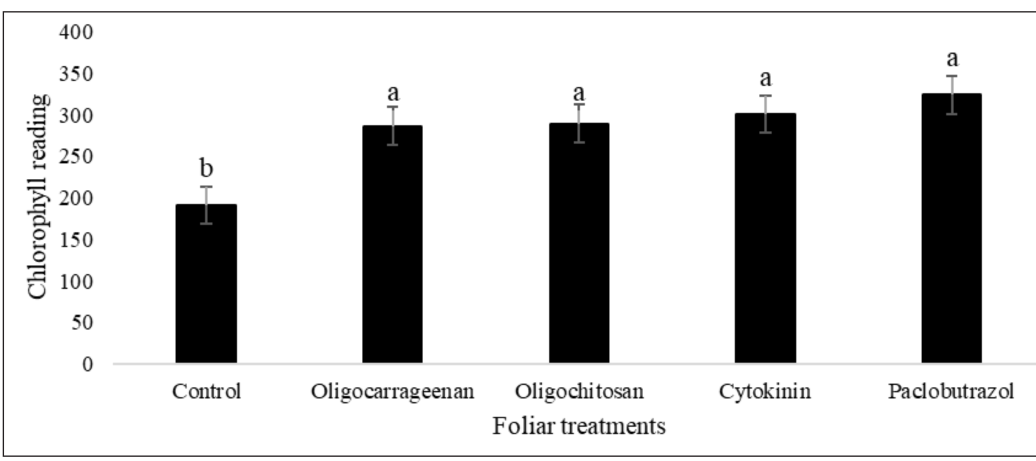

Figure 6. Effects of biostimulants and PGRs on the chlorophyll index of cacao shade leaves at 3 MAT based on the FieldScout CM 1000 under irrigated condition
cv (%)= 7.91; p= .0007**
Treatment means with the same superscript are not significant according to the Tukey's HSD test (p ≤.01)

Cacao Flowering in Response to Biostimulants and PGRs Under Different Irrigation Practices

Cushions

The percentage change in number of cushions in cacao was not significantly influenced by the interaction between irrigation practice and foliar treatments. At 4MAT, there was a significant (p= .0425) difference observed in the percentage change in number of cushions within cacao trees in response to foliar treatments under the irrigated experiment (Figure 7) but not in the rainfed condition. The highest percent change in number of cushions at 4MAT (370.24%) was notable on cacao trees applied with oligochitosan and was comparable to that of cacao trees applied with cytokinin (264.71%), paclobutrazol (277.72%), and the control (291.09). The oligocarrageenan treatment had resulted in lower percentage change in the number of cushions (228.78%) at 4MAT but was comparable to those of cytokinin, paclobutrazol, and control treatments under the irrigated experiment.

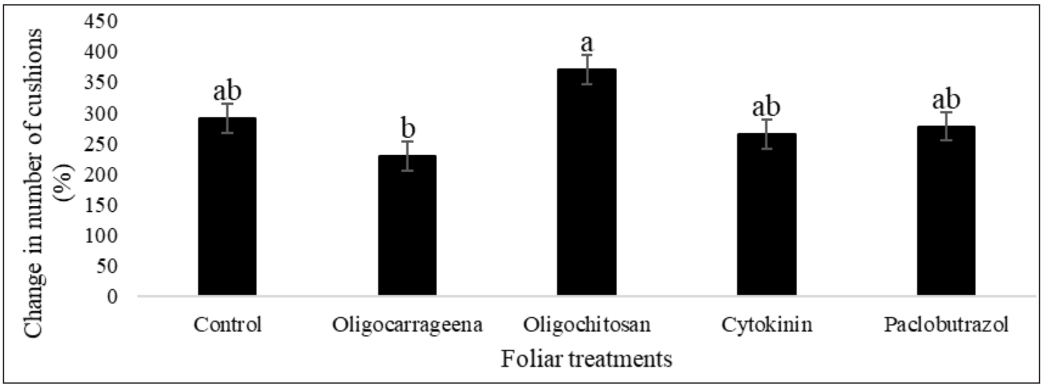

Figure 7. Percent change in number of cushions in cacao trees at 4MAT in response to foliar treatments under the irrigated condition
cv (%)= 13.38; p= 0.0192*
Treatment means with the same superscript are not significant according to the Tukey's HSD test ($p \leq .05$)

Flowers

The interaction between irrigation practice and foliar treatment was not significant on the percentage change in the number of flowers. The different foliar treatments, however, exhibited significant differences in terms of percentage change in the number of flowers formed at 4 MAT in the rainfed condition (Figure 8) and not observed in the irrigated experiment. Oligochitosan foliar spray had resulted in higher percentage change in the number of flowers but was comparable to those treated with oligocarrageenan, cytokinin, and paclobutrazol (Figure 8). The control trees exhibited lower percentage change in the number of flowers at 4MAT (Figure 8).

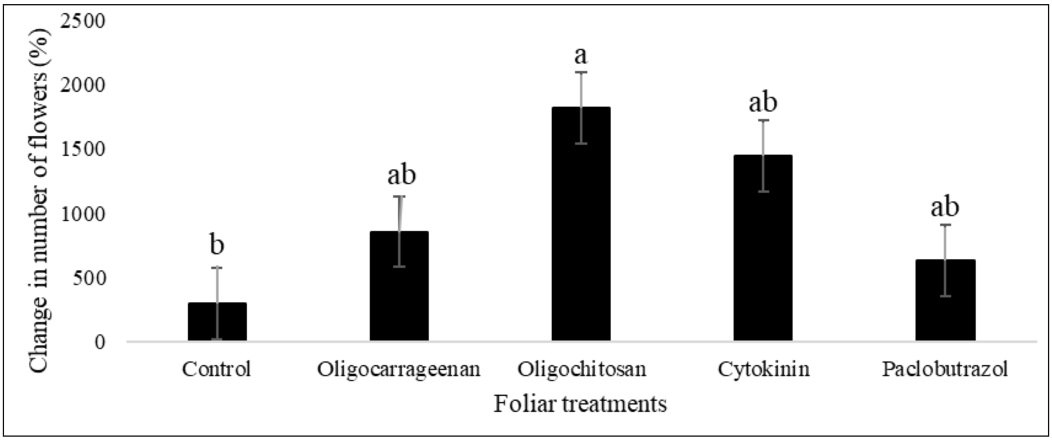

Figure 8. Effects of foliar treatments on the percentage change in the number of flowers in cacao trees at 4MAT under the rainfed condition
cv (%)= 49.51; *p*= .0325*
Treatment means with the same superscript are not significant according to the Tukey's HSD test ($p \leq .05$)

Effects of Biostimulants and PGRs on Cherelle Wilt Incidence and Pod Retention under Different Irrigation Practices

The cherelle wilt and pod retention were not influenced by the interaction between the irrigation practice and foliar treatment. The effects of biostimulants and PGRs on cherelle wilt incidence and pod retention were only significant under the irrigated condition and not observed in the rainfed experiment (Table 1). High incidence of cherelle wilt (68.50%) was observed from the control trees. In contrast, lower incidence of cherelle wilt (37.52%) was noted from trees treated with paclobutrazol. The cherelle wilt incidence also seems to have decreased considerably with cytokinin treatment but the effect was statistically comparable with the control (Table 1).

Table 1
Cherelle wilt incidence and pod retention of cacao trees in response to biostimulants and PGRs under the irrigated experiment

Foliar treatment	Cherelle wilt incidence (%)	Pod retention (%)
Control	68.50 ± 4.48^{a}	50.80 ± 21.08^{b}
Oligocarrageenan	56.47 ± 16.98^{ab}	55.03 ± 23.22^{ab}
Oligochitosan	54.96 ± 14.58^{ab}	47.32 ± 25.56^{b}
Cytokinin	43.98 ± 0.84^{ab}	85.03 ± 24.22^{a}
Paclobutrazol	37.52 ± 9.48^{c}	70.46 ± 24.47^{ab}
cv (%)	19.08	18.67
Pr (>F)	.0372*	.0148*

Treatment means within column with same superscript are not significant according to the Tukey's HSD test ($p \leq .05$)

The highest pod retention (85.03%) under the irrigated experiment was recorded in cytokinin-treated cacao trees. Lower pod retention was observed in cacao trees in the oligochitosan and control treatments with 47.32, and 50.80 % respectively (Table 1).

Effects of Biostimulants and PGRs on the Seed Fresh Weight of Cacao Under Different Irrigation Practices

Significant (*p*= .0280) interaction between the irrigation practice and foliar treatment on seed fresh weight of cacao was observed (Figure 9). Under the rainfed condition, lighter fresh seed weight was recorded from cacao in the control. Under the irrigated condition, the fresh seed weight of cacao in the control did not vary with the cacao applied with different foliar treatments (Figure 9).

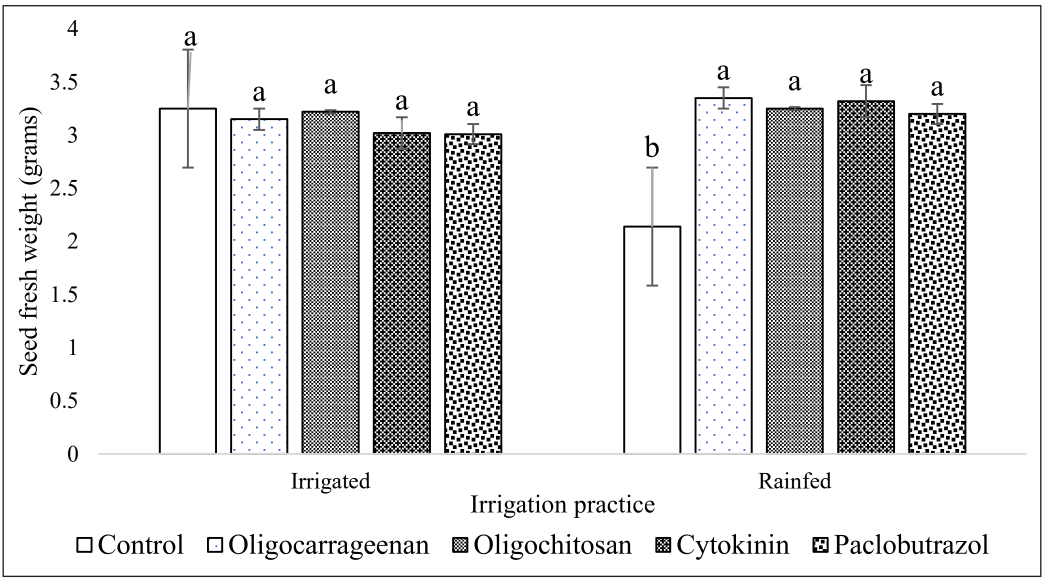

Figure 9. Interaction effect between the irrigation practice and foliar treatment on the mean seed fresh weight of cacao
cv (%)= 12.25; *p*= .0280*
Treatment means with the same superscript within same irrigation practice are not significant according to the Tukey's HSD test (p ≤.05)

Effects of Biostimulants and PGRs on the Production of Full Beans and Flat Beans Under Different Irrigation Practices

The interaction between irrigation practice and foliar treatment on the production of full beans and flat beans of a cacao tree was not significant (Table 2). Significant differences on the full beans and flat beans production of a cacao tree were however exhibited by those foliar treatments. As shown in Table 2, control trees produced lesser full beans and more flat

Table 2
Full beans and flat beans production of a cacao tree in response to biostimulants and PGRs under different irrigation practices

Foliar treatment (Fol)	Irrigation practice (IP)	Full beans (%) based on weight		Flat beans (%) based on weight		Full beans (%) based on number		Flat beans (%) based on number	
		$\bar{x}_1$	$\bar{x}_2$	$\bar{x}_1$	$\bar{x}_2$	$\bar{x}_1$	$\bar{x}_2$	$\bar{x}_1$	$\bar{x}_2$
Control	Irrigated	94.84±1.3	$94.54±2.5^{b}$	5.16±1.3	$5.46±2.5^{a}$	85.26±1.0	$83.87±6.9^{b}$	14.74±1.0	$16.13±6.9^{a}$
Control	Rainfed	94.24±3.5		5.76±3.5		82.48±8.8		17.52±8.8	
Oligocarrageenan	Irrigated	98.95±1.1	$98.82±2.6^{a}$	1.05±1.1	$1.18±2.6^{b}$	97.11±2.4	$94.04±6.8^{a}$	2.89±2.4	$5.96±6.8^{b}$
Oligocarrageenan	Rainfed	98.69±0.8		1.31±0.8		90.97±7.5		9.03±7.5	
Oligochitosan	Irrigated	99.19±0.5	$98.71±2.6^{a}$	0.81±0.5	$1.29±2.6^{b}$	97.01±2.4	$96.04±6.8^{a}$	2.99±2.4	$3.96±6.8^{b}$
Oligochitosan	Rainfed	98.23±1.9		1.77±1.9		95.07±5.2		4.93±5.2	
Cytokinin	Irrigated	97.88±1.0	$98.29±2.2^{a}$	2.12±1.0	$1.71±2.2^{b}$	94.84±1.7	$94.95±6.6^{a}$	5.16±1.7	$5.05±6.6^{b}$
Cytokinin	Rainfed	98.70±0.5		1.30±0.5		95.06±1.7		4.94±1.7	
Paclobutrazol	Irrigated	97.54±1.6	$97.38±2.2^{a}$	2.46±1.6	$2.62±2.2^{b}$	90.31±4.3	$90.23±6.4^{a}$	9.69±4.3	$9.77±6.4^{b}$
Paclobutrazol	Rainfed	97.23±1.3		2.77±1.3		90.16±2.7		9.84±2.7	
cv (%)		1.65	1.65	65.78	65.78	5.17	5.17	58.09	58.09
Pr (>F)	IP	$.6547^{ns}$	-	$.6547^{ns}$	-	$.1686^{ns}$	-	$.1686^{ns}$	-
	Fol	.0016**	.0016**	.0016**	.0016**	.0026**	.0026**	.0026**	.0026**
	IP:Fol	$.9008^{ns}$	-	$.9008^{ns}$	-	$.7829^{ns}$	-	$.7829^{ns}$	-

$\bar{x}_{1\text{-}}$Means of foliar treatment in irrigated and rainfed experiments, and irrigation practice for the same foliar treatment are not significant according to Tukey's HSD test
$\bar{x}_{2\text{-}}$ Superscript represent the significance between foliar treatment means within the column according to the Tukey's HSD test
ns- not significant
**- significant at $p \leq .01$

beans. This situation was reversed by the foliar spray of biostimulants (oligocarrageenan and oligochitosan) and PGRs (cytokinin and PBZ).

Effects of Biostimulants and PGRs on the Dried Bean Weight of Cacao under Different Irrigation Practices

There was no significant interaction between irrigation practice and foliar treatments on the dried bean weight of cacao. The dried bean, however, was significantly (*p*= .0287) influenced by the foliar treatment (Figure 10). The cacao in the control treatment produced lighter dried beans (1.35 g). Foliar spray of oligocarrageenan resulted in heavier dried beans (1.50 g) of cacao as shown in Figure 10.

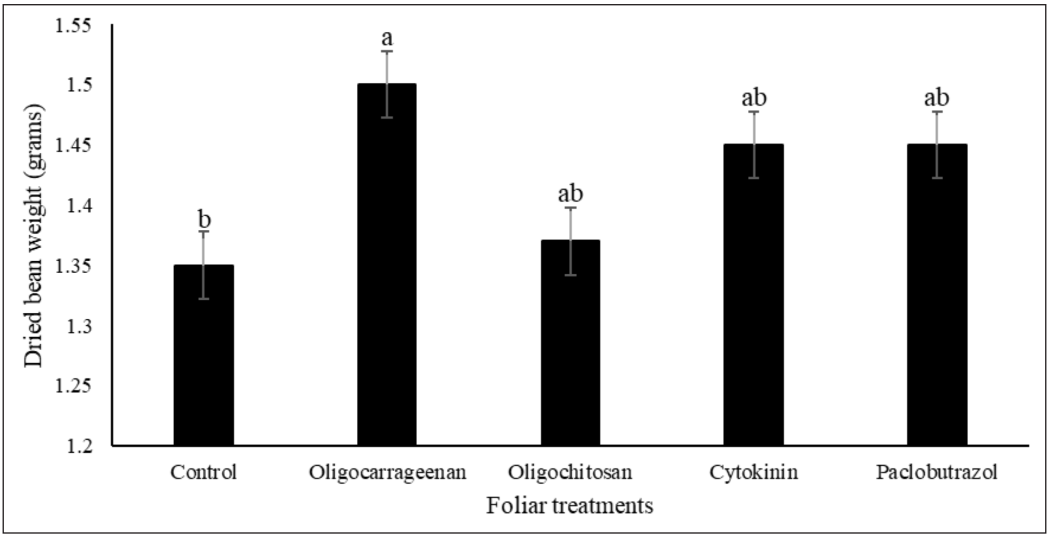

Figure 10. Average dried bean weight of cacao in response to foliar treatments
cv (%)= 5.85; *p*= .0287*
Treatment means with the same superscript are not significant according to the Tukey's HSD test ($p \leq .05$)

Effects of Biostimulants and PGRs on the Bean Count of Cacao under Different Irrigation Practices

There was no significant interaction observed between irrigation practice and foliar treatment on the bean count of cacao. Rather, the bean count of cacao was significantly (*p*= .0378) influenced by the foliar treatment (Figure 11). Highest bean count, meaning lighter beans, was recorded in the control trees with 74.42. In contrast, the oligocarrageenan foliar spray resulted in lowest or better bean count (67.50).

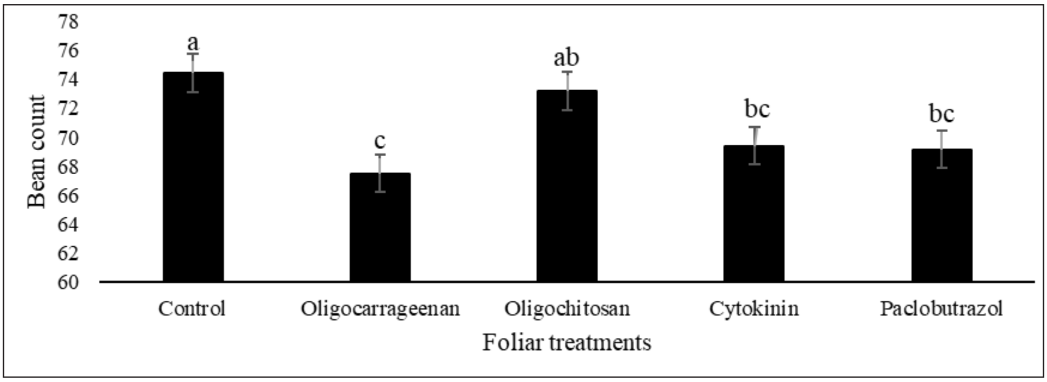

Figure 11. Bean count of cacao in response to foliar treatments
cv (%)= 5.60; *p*= .0378*
Treatment means with the same superscript are not significant according to the Tukey's HSD test (p ≤.05)

Dry Weight per Tree of Marketable Beans in Response to Biostimulants and PGRs

The dry weight (DW) per tree of marketable beans considered here was only for one month (May) harvest. The irrigation practice during the dry season did not affect the efficacy of biostimulants and PGRs on the dried weight of marketable beans in cacao. Foliar treatments, however, influenced the DW per tree of 'UF 18' cacao. Heavier DW per tree of marketable beans was achieved through the foliar spray of cytokinin, paclobutrazol, and oligocarrageenan (Figure 12). This is comparable to the DW per tree of marketable beans of oligochitosan-treated trees. The control trees had the lowest yield per tree but was comparable with those of oligochitosan-treated trees (Figure 12).

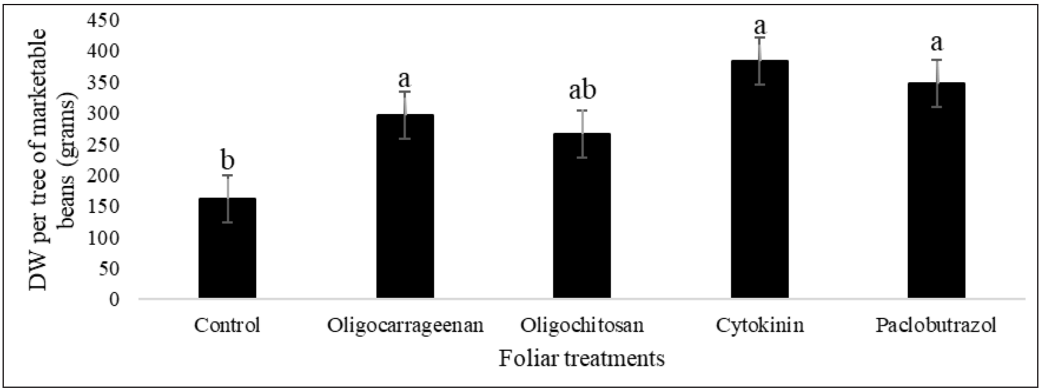

Figure 12. Dry weight per tree of marketable beans
cv (%)= 33.87; *p*= .0151*
Treatment means with the same superscript are not significant according to the Tukey's HSD test (p ≤.05)

Yield per Tree per Year of Cacao in Response to Biostimulants and PGRs under Bukidnon, Philippines

The estimated yield per tree per year of cacao is shown in Table 3. During the wet season (irrigated), the yield per tree estimate of the control trees is doubled through the cytokinin foliar spray. However, foliar spray of biostimulants and PGRs during the dry season (rainfed) did not cause a significant yield difference compared to the control. The foliar spray of cytokinin, paclobutrazol, and oligocarrageenan, however, showed to be promising in producing higher yield per tree per year with 2.6226, 2.3614, and 1.9834 kg respectively compared to the control with only 1.1120 kg. Likewise, oligochitosan resulted in a slight increase of yield per tree per year versus the control.

Table 3
Estimated yield per tree per year of cacao in response to biostimulants and PGRs under Bukidnon, Philippines condition

Foliar treatment	Yield per tree per season (kg)		Yield per tree per year (kg)	Yield difference versus the control (kg)
	Dry (rainfed)	Wet (irrigated)		
Control	0.3746±0.231	0.7374±0.132[b]	1.1120±0.080[c]	-
Oligocarrageenan	1.1485±0.120	0.8349±0.189[ab]	1.9834±0.628[ab]	0.8714
Oligochitosan	0.9753±0.773	0.8108±0.136[ab]	1.7861±0.471[b]	0.6741
Cytokinin	1.0016±0.169	1.6210±0.302[a]	2.6226±0.004[a]	1.5106
Paclobutrazol	1.0574±0.511	1.3040±0.413[ab]	2.3614±0.258[ab]	1.2494
cv (%)	38.19	28.70	17.93	-
P (>F)	.1424[ns]	.0290*	.0065**	-

[ns]- not significant; *- significant at p ≤.05; **- significant at p ≤.01
Treatment means with the same superscript are not significant according to the Tukey's HSD test

DISCUSSION

Cacao trees can be cultivated as a monocrop and an intercrop to existing perennial crops such as coconut and banana. But the latter is ideal especially during the dry season as it creates microclimate that minimise evapotranspiration to retain more cacao pods per tree. However, not all local farmers venturing cacao production employs intercropping. Supplemental irrigation during the dry season may be an alternative for a monocrop cacao plantation, however, this practice is costly and labourious. Thus, biostimulants and plant growth regulators (PGRs) that will increase pod retention and yield per tree in monocropped cacao plantations during water-limiting condition or during the dry season in the Type III climate of the country is a potential solution, hence this study.

The biostimulants tested in this study are the oligocarrageenan and oligochitosan, as these two are gaining popularity nowadays. The oligocarrageenan and oligochitosan biostimulants have been reported to reduce the negative impact of limited water conditions in various crops by stimulating some plant physiological processes that improved plant growth and yield (Moenne & Gonzalez, 2021; Thye et al., 2022; Bongalos et al., 2019; Vicena et al., 2024). On the other hand, the PGRs used in this study are cytokinin and PBZ. Cytokinin is a phytohormone which prevents degradation of leaf chlorophyll especially during abiotic stress conditions. Also, PBZ application has been reported to increase cytokinin levels according to several studies cited by Desta and Amare (2021). Foliar applications of these PGRs could improve nutrient uptake as well as photosynthesis of cacao during the dry season.

Chlorophyll is the green pigment in the chloroplast responsible for capturing photosynthetically active radiation (Mandal & Dutta, 2020). Thus, plants with higher leaf chlorophyll content presumably have better photosynthetic efficiency. Generally, the foliar applications of biostimulants and PGRs increased the chlorophyll index of cacao leaves in this study. This finding is supported by numerous reports that oligocarrageenan and oligochitosan increased the chlorophyll content of leaves which allow plants to synthesise more photoassimilates (Abad, Dean et al., 2018; Chen et al., 2023; Hossain et al., 2024; Khalil & Eldin, 2021; Li et al., 2021; Naeem et al., 2014; Safikhan et al., 2018; Shukla et al., 2016; Thye et al., 2022) that are needed especially by the developing fruits. Similarly, cytokinin and PBZ are PGRs that enhance chlorophyll biosynthesis in plant leaves as well as preventing chlorophyll degradation (Desta & Amare, 2021; Fathy et al., 2022; Fletcher et al., 1982; Liu et al., 2022; Solichatun et al., 2021; Tesfahun, 2018; Xu & Huang, 2017) which supports the result of this study.

It has been reported that oligochitosan enhances the flowering intensity of plants (Ahmed et al., 2016; Sultana et al., 2017) which conforms to the result of this study. In any fruit crop production, however, fruit setting is more important than just flowers. Once cacao flowers are successfully pollinated, these will become cherelles. The young cherelles (<BBCH 75) are susceptible to wilting especially when there is a limited supply of photoassimilates within a tree. In this study, the incidence of cherelle wilt was reduced through the foliar spray of paclobutrazol. Moreover, cytokinin resulted in higher retention of cacao pods which corroborates to the report of Kumara et al. (2023) that cytokinin enhances fruit retention in mango trees.

The aim of tailoring horticultural technology in fruit and plantation crops production is to improve yield and quality. This study reveals that the average weight of fresh cacao bean was lighter in experiment set-up without supplemental irrigation during the dry season. This condition was reversed with the foliar spray of biostimulants and PGRs. Among foliar treatments, oligocarrageenan resulted in the production of heavier dried beans. Moreover, foliar spray of biostimulants and PGRs resulted in better bean count, with more full beans, and lesser flat beans than the control.

Cacao dried beans are the raw material in making chocolates. In the Philippines, the target yield per tree per year of cacao is set at 2 kg. However, average production ranges only from 0.5 to 1.0 kg per tree per year (DA, 2017) despite the use of high-yielding grafted cacao clones. In this study, the estimated yield per tree per year of cacao not applied with biostimulants or PGRs is at 0.82 kg. The foliar spray of oligocarrageenan, oligochitosan, cytokinin, and paclobutrazol exhibited as a promising solution to improve the yield per tree per year of 'UF 18' cacao by ~0.8714, ~0.6741, ~1.5106, and ~1.2494 kg of dried beans, respectively under Bukidnon, Philippines condition.

Unlike fertilisers and phytohormones, biostimulants do not directly supply nutrients and hormonal needs of crops (Gupta et al., 2023). Instead, biostimulants stimulate plant physiological processes that enhance nutrient uptake and accumulation of dry matter and eventually improve crop yield (Jardin, 2015) especially under abiotic stress conditions (Li et al., 2022). Numerous reports corroborate the findings of this present study that oligocarrageenan foliar spray improves crop yield. For example, oligocarrageenan had improved the yield of peanut (Abad, Aurigue et al., 2018), mungbean (Gatan et al., 2019), and coffee (San et al., 2020) which supports the findings of this present study. On the other hand, PGRs are classic compounds used to improve crop yield under abiotic stress such as water-limiting conditions. These compounds directly regulate the plant's endogenous hormone level that led to an accumulation of more photoassimilates and thereby increasing dry matter and yield of crops (Sosnowski et al., 2023). Among the phytohormones, cytokinin treatment is proven to improve yield of crops as this hormone is responsible for maintaining leaf chlorophyll, enriching plant nutrients, cell division, and have a strong impact on crop yield (Mandal et al., 2022; Prasad, 2022; Sharma et al., 2022). These recognised effects of cytokinin in plants might have also occurred in this present study, thus improving cacao yield performance especially during the dry season in Bukidnon, Philippines. In comparison, paclobutrazol has been well-studied and known to improve the yield of mango (Yeshitela et al., 2004) and cacao (Nieves et al., 2024) trees. Several studies cited in the review of Desta and Amare (2021) have shown that the paclobutrazol application resulted in increased levels of cytokinin and regulates the sink-source relationships of trees, thus this PGR also improves the yield of cacao in this present study.

CONCLUSION AND RECOMMENDATION

This is the first report that the biostimulants (oligocarrageenan and oligochitosan) are bioactive in cacao and positively influenced the reproductive characters of cacao under water stress. Likewise, PGRs (cytokinin and PBZ) also improved the reproductive characters of cacao during water stress. Among the foliar treatments, oligochitosan exhibited potential to increase cacao flowering intensity. Cherelle wilt incidence is reduced by paclobutrazol treatment, while cytokinin increased the pod retention of cacao. Generally, the biostimulants and PGRs foliar spray improved most of the yield parameters of cacao measured in this study during the dry season in Bukidnon, Philippines. Among the biostimulants and PGRs tested, oligocarrageenan, cytokinin, and paclobutrazol were noteworthy in the production of heavier dry weight per tree of marketable beans. The foliar spray of biostimulants and PGRs resulted in significant increase versus the control in terms of yield per tree per year of cacao. Thus, foliar sprays of these biostimulants and PGRs are recommended to boost cacao production during the dry season.

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