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TROPICAL AGRICULTURAL SCIENCE

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Pertanika Journal of Tropical Agricultural Science
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PERTANIKA JOURNAL OF TROPICAL AGRICULTURAL SCIENCE

About the Journal

Overview

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

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

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

The journal is available world-wide.

Aims and Scope

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

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

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To publish journals of international repute.

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

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1. The journal's Chief Executive Editor and Editor-in-Chief examine the paper to determine whether it is relevance to the journal's needs in terms of novelty, impact, design, procedure, language as well as presentation and allow it to proceed to the reviewing process. If it is 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 to strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the research field.

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

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 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 the 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.

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Pertanika Journal of Tropical Agricultural Science
Vol. 49 (2) Apr. 2026

Contents

Foreword i
Md Kamal Uddin

Morphological Variation of Liberica Coffee (*Coffea liberica* Hiern) in Kalipuro Sub-district, Banyuwangi Regency 449
Naila Izzatul Mukhoyyaroh, Rodiyati Azrianingsih, Estri Laras Arumingtyas, and Luchman Hakim

Analogue Rice from *Amorphophallus muelleri* and Potato with Enhanced Nutritional Properties: Lower Glycemic Index and Higher Fibre Content 473
Kisroh Dwiyono, Nor Elliza Tajidin, Maman Abdurachman Djauhari, Elisa Azura Azman, Nur Syahirah Abdul Rashid, Viktor Vekky Ronald Repi, and Noverita

The Influence of Zinc and Boron Application on Growth, Yield, Seed Quality, and Nutrient Content in Non-hybrid Corn 487
Agustiansyah, Paul Benyamin Timotiwu, Eko Pramono, Galuh M. Pramudyano, and David Chandra

A Second Life for Agro-industrial Residues: Edible Coating from Roselle Decoction Residues for Preserving Wax Apple Quality and Antioxidant Properties 497
Nur Amirah Yusoff, Fauziah Tufail Ahmad, Aidilla Mubarak, Razifah Mohd Razali, and Husni Hayati Mohd Rafdi

Behavioural Response of Cabbage White Butterfly (*Pieris rapae*) as Affected by Different Levels of Deltamethrin Insecticide 519
Jackie Lou F. Bagote, Jenny S. Palor, Angelo DG. Rullepa, Mark Argyll D. Tambaoan, and Schereid Joi N. Ugnasi

Storage Reserve Composition and Storage Behaviour of *Amorphophallus muelleri* Blume Seed 537
Siti Fadhilah, Eny Widajati, Satriyas Ilyas, Endah Retno Palupi, and Abdul Qadir

Synergistic Application of Marine-derived Actinomycetes and Microalgae to Enhance Rice Seedling Growth and Salinity Stress Tolerance 555
Alimuddin Ali, Muhammad Junda, Nur Haedar, Hasliana, Didik Imam Sakirin, and Herlina Rante

Using Spectral Vegetation Indices to Study the Growth of *Opuntia ficus-indica* and Identify Suitable Production Sites in the Southwestern Region of Saudi Arabia 587
Abdulmalek A. Muharram, Elkamil Tola, Rashid S. Al-Obeed, Fahad Al-Qurainy, Khalid A. Al-Gaadi, Rangaswamy Madugundu, Ahmed M. Zeyada, Mohamed K. Edrris, Nabil Mohammed, and Abdulhakim A. Aldubai

Early Performance Results of Polyhalite on Newly Planted Oil Palms 607
Ng Fang Wen, Siti Hajar, and Lim Jing Jie

Valorisation of Kopyor Coconut Waste for Fatty Acid Production by *Yarrowia lipolytica* 627
Firda Dimawarnita, Muhammad Fayyadh Altamis Erwinsyah, Puspo Edi Giriwono, Yora Faramitha, Irma Kresnawaty, Urip Perwitasari, Titin Haryati, and Ario Betha Juanssilfero

Phenological Growth Stages of *Artocarpus odoratissimus* using the Extended BBCH Scale and its Applications in Determining Optimal Harvest Maturity 647
Isniti Richard, Halifah Afiza Ismail, Shiamala Devi Ramaiya, Gerevieve Bangi Sujang, Nuratika Tamimi Sheikh Mohamed, and Kundan Kishore

Enhancing the Physical Properties of Egg Custard-based Diets through Feed Supplements to Improve the Growth Performance of *Macrobrachium rosenbergii* Larvae 665
Riyad Taguemount, Jarunan Pratoomyot, Andrew P Shinn, and Rasina Rasid

Review Article 689
A Review of Key Factors Influencing Forest Fire and Wildfire Susceptibility: The Role of Climatic, Environmental, Topographic, and Anthropogenic Variables
Yee Jian Chew, Shih Yin Ooi, Ying Han Pang, and Zheng You Lim

Morphological Differences of Experimental Hybrids and Check Varieties of High-yielding Grain Maize 715
Siti Norfaroha Ariffin, Juju Nakasha Jaafar, Ghizan B. Saleh, Nazatul Shima Naharudin, and Samsuri A. Wahid

Unravelling the Diversity of *Azolla cristata* from the Progo and Opak River Basins based on Inter-simple Sequence Repeat Marker and Morphological Analysis 733
Nafiah Khoirunnisa, Adib Fakhruddin Yusuf, Aji Sukma Iqbal Najibulloh, Abdul Razaq Chasani, Rina Sri Kasiamdari, and Budi Setiadi Daryono

Review Article 761
Reproduction Technology for Sustainable Portunid Crab's Aquaculture: A Review
Ariffin Hidir, Mohd Amran Aaqillah-Amr, and Mhd Ikhwanuddin

Selangor Public Perceptions of Neutering and Preventive Health Measures for the Health of Cats and Dogs 775
Nur Athirah Nabila Jamaluddin, Michelle Fong Wai Cheng, Tengku Rinalfi Putra Tengku Azizan, Muhd Khaizer Omar, and Syamira Syazuana Zaini

Review Article
The Ecology and Conservation of Orchids in Ultramafic Habitats of Kinabalu Park, Sabah, Malaysia 791
Harniza Izzati Razali and Siti Fatimah Md.-Isa

Review Article
A Review on the Optimisation of Enzymatic Treatment in Tropical Fruit Juice: Impacts on Physicochemical and Functional Properties 841
Hasdarahayu Mohd Hassan, Mohd Azrie Awang, Ahmad Hazim Abdul Aziz, Asep Awaludin Prihanto, Abdul Aziz Jaziri, and Siti Faridah Mohd Amin

Abscisic Acid Exogenous Spraying to Improve Sorghum (*Sorghum bicolor* (L.) Moench) Genotypes for Tolerance to Abiotic Stress 863
Abdullah Amar Saleh, Yaseen Obaid Noori Ahmed Sharif, and Ali Hussein Raheem

Diversity and Ecological Insights of Fruit Bats in Durian Orchards of Sarawak, Malaysian Borneo 881
Sara Dalton Saging, Muhd Amsyari Morni, Wong Sin Yeng, Abd Rahman Mohd-Ridwan, Faisal Ali Anwarali Khan, and Jayasilan Mohd-Azlan

Phylogeography and Genetic Structure of the Giant Freshwater Prawn (Decapoda: Palaemonidae) in Peninsular Malaysia using Mitochondrial DNA Analysis 901
Dania Aziz, Afrina Irdina Roszizul Kahairi, Athirah Mohd Bakri, Nur Syuhada Mohd Fuad, Izzah 'Atirah Mohd Hir Tarmidi, Yuzine Esa, Md. Lifat Rahi, and Muhammad Dailami

Application of Multi-level Factorial Design in Optimisation of Ultrasound-assisted Extraction (UAE) Condition from *Erythrina variegata* L. Leaves on Antioxidant Activities 919
Nur Fazira Abdul Rahim, Norhayati Muhammad, Fazleen Izzany Abu Bakar, Mohd Fadzelly Abu Bakar, Siti Fatimah Sabran, Furzani Pa'ee, Balkis A. Talip, Ain Zamira Muhamad Zamri, Rozaliana Ab Karim, Nur Zainatul Nadra Zainol, Aliff Hisyam A Razak, and Rabiah Ahmad

Review Article 937
Life Cycle and Feeding Behaviour of Rice Stem Borer Species in Southeast Asian Regions' Paddy Fields
Aisyah Pratiwi Suryanto Sugian, Patricia King Jie Hung, Muhamad Azmi Mohammed, Tunung Robin, Leong Sui Sien, Freddy Yeo Kuok San, Zakry Fitri Ab. Aziz, Noorasmah Saupi, Shiamala Devi Ramaiya, Ribka Alan, Adrian Daud, Anita Rosli, Philip Lepun, Zahora Ismail, Sivasangar Seenivasagam, and Suziana Hassan

Effect of Boron Fertilisation and Trisodium Phosphate (TSP) Priming on Fruit and Seed Quality of GM-2 Tomato (*Solanum lycopersicum* L.) 967
Amira Mufida Zharfa, Rudi Hari Murti, and Budiastuti Kurniasih

Optimising Glucomannan Production in *Amorphophallus oncophyllus* by Varying Tuber Size and Fertiliser Source 985
F. Deru Dewanti, Ramdan Hidayat, Careca Sepdihan Rahmat Hidayatullah, and Oentari Prilaningrum Sutanto

Drought Tolerance of Five Selected Rice (*Oryza sativa* L.) Genotypes based on Morphological Physio-biochemical and Phenotypic Polymorphism 997
Noor Asmaa Awang, Mohd Razi Ismail, and Zulkarami Berahim

Foreword

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

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

This issue contains 27 articles: five review articles and the rest are regular articles. The authors of these articles come from different countries namely Bangladesh, China, India, Indonesia, Iraq, Malaysia, Philippines, Saudi Arabia, and Thailand.

A selected article entitled "Behavioural Response of Cabbage White Butterfly (*Pieris rapae*) as Affected by Different Levels of Deltamethrin Insecticide" investigates the impact of sub-lethal and lethal insecticide exposure on the movement and feeding patterns of a major cruciferous pest. Using a dual-container setup and a "one-way no-choice method," the researchers evaluated larval survival, distance travelled, and leaf area damage under varying deltamethrin coverage levels. The findings revealed that while 100% coverage effectively induced high mortality, partial coverage led to behavioural changes, such as increased movement, as larvae attempted to avoid treated surfaces. This study underscores the importance of achieving uniform insecticide application to prevent pest dispersal and ensure effective crop protection. Further details of this study can be found on page 519.

The study entitled "Early Performance Results of Polyhalite on Newly Planted Oil Palms" explores the efficacy of a natural multi-nutrient mineral as a sustainable alternative to conventional fertilisation programs. Conducted in a Malaysian oil palm estate, the study evaluated the vegetative growth performance and cost-efficiency of Polyhalite, which provides essential potassium, calcium, magnesium, and sulfur. Results demonstrated that incorporating 0.3 kg of Polyhalite into planting holes during transplanting delivered vegetative growth comparable to conventional practices while remaining more cost-effective. The study suggests that Polyhalite serves as a viable slow-release fertiliser that can help mitigate labour shortages by reducing application rounds and minimising environmental risks associated with polymer-coated fertilisers. Full details of this study are available on page 607.

The review article titled “Life Cycle and Feeding Behaviour of Rice Stem Borer Species in Southeast Asian Regions Paddy Fields” provides a comprehensive synthesis of the biological and ecological characteristics of six major rice stem borer species. The authors correlate the distinct egg-laying behaviours, feeding sites, and infestation timings of species such as *Scirpophaga incertulas, Scirpophaga innotata, Chilo suppressalis, Chilo polychrysus, Chilo auricilius, and Sesamia inferens* with rice phenology to explain variations in damage symptoms like “deadheart” and “whitehead”. By identifying critical research gaps in economic thresholds across Southeast Asia, the review advocates for an ecology-aligned Integrated Pest Management (IPM) framework. This strategic alignment aims to enhance the precision of control measures, such as biological agents and cultural practices, thereby reducing the agricultural sector’s reliance on broad-spectrum insecticides. Further detailed information can be found on page 937.

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

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

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

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

Editor-in-Chief
Md Kamal Uddin

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Morphological Variation of Liberica Coffee (*Coffea liberica* Hiern) in Kalipuro Sub-district, Banyuwangi Regency

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ABSTRACT

Coffea liberica has encountered challenges related to infraspecific taxa, leading to confusion and difficulty in taxonomic identification. This study aims to determine the Liberica coffee variant in the Kalipuro Sub-district based on morphological traits, to clarify whether it is more closely related to *Coffea liberica* var. *liberica* or *Coffea liberica* var. *dewevrei*. A field study was conducted to identify reference morphological traits used as the basis for grouping Liberica coffee variants. Quantitative and qualitative characteristics of the vegetative and reproductive phases were assessed and recorded. *Coffea liberica* exhibits four representative branch types: drooping with upward-curving tips, horizontal, declinate (drooping branch), and leaning upward. The study discovered that Liberica coffee in Kalipuro varies in leaf shape, colour, margins, apex, fruit colour, bean colour, and shape. However, some traits showed relatively low variability among accessions, and inconsistencies remained. Therefore, key characters i.e., leaf surface and shape index, number of flowers/inflorescences, fruit volume, bean shape, were used to determine the taxonomic position of Liberica coffee. The variety found in the Kalipuro more closely resembled *C. liberica* var. *dewevrei* than *C. liberica* var. *liberica,* based on a Jaccard similarity index of 0.6. Nevertheless, additional genetic research is required to clarify the taxonomic status of Liberica coffee in Kalipuro, as numerous shared characteristics lead to ambiguity among these varieties.

Keywords: Accession, coffee, characterisation, liberoid, morphological trait

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INTRODUCTION

Coffea is one of the most economically important genera among over 500 genera in

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the Rubiaceae family. *Coffea* comprises over 120 species, with *Coffea arabica* and *Coffea canephora* being the most commercially popular worldwide (Davis et al., 2011). Coffee is a major export commodity cultivated in approximately 80 countries, supporting the livelihoods of over 20 million people worldwide through production and related industries. It is produced either through a traditional system, wild harvesting, or managed agroforestry systems involving the removal of undergrowth and canopy. Commercial coffee species in Indonesia originate from Africa (Ayalew, 2018). Coffee is among Indonesia's leading commodities, alongside palm oil, rubber, cocoa, tea, and coconut. Indonesia supplies 7% of the global coffee demand (Direktorat Jendral Perkebunan of Indonesia, 2019). In 2021, it ranked as the fourth-largest coffee producer globally, after Brazil, Vietnam, and Colombia. In 2023, annual production reached 758,725 tons, with a productivity rate of 789 kg/ha (BPS Indonesia, 2024).

Since the Dutch colonial era, Banyuwangi Regency, especially the Slope of Mount Ijen, has been Indonesia's major coffee-producing region. In this region, the population of *C. liberica* remains significant alongside *C. arabica* and *C. canephora*, contributing to local coffee production. Coffee in Banyuwangi is produced through both large coffee plantations managed by state and private companies, and smallholder plantations owned and managed by local communities. In 2020, average coffee production—particularly Robusta—reached 876 kg/ha, the highest in East Java Province (Direktorat Jendral Perkebunan of Indonesia, 2021). With the expansion of coffee cultivation on Java Island, Banyuwangi Regency, specifically Kalipuro Sub-district, has become one of the areas with the high *Coffea* diversity. The primary crops planted by the local communities are *Coffea canephora* and *Coffea liberica* (Hakim et al., 2022).

For several years, *C. liberica* has faced infraspecific taxonomic issues. Although classified as a single species with two varieties— *Coffea liberica* var. *liberica* and *Coffea liberica* var. *dewevrei*—its taxonomy remains a source of confusion (Baltazar & Buot, 2019). The high diversity of Liberica coffee, combined with farmers' limited knowledge and ability to identify its variants, poses a challenge in accurately determining the taxa of *Coffea liberica* variants. Moreover, the difficulty in determining the taxa of Liberica coffee variants arises from a limited understanding of their morphological characteristics. As a result, beans from different varieties are often harvested and mixed, hindering proper selection. Resolving this taxonomic ambiguity is essential to improve classification accuracy and enhance the product's market value.

As one of the nation's coffee production centres, Kalipuro Sub-district holds unexplored potential for Liberica coffee diversity. Morphological variation observed in agricultural fields allows the examination of seed origin and environmental conditions as factors influencing this diversity. Such variants are determined by genetic variation and environmental factors (Wei et al., 2025). Morphologically, variants of *C. liberica* exhibit

differences that can be identified through detailed morphological analysis and subsequently confirmed through molecular analysis. In Kalipuro Sub-district, uncertainty exists regarding the classification of the local Liberica coffee variant, specifically whether it is more closely related to *C. liberica* var. *liberica* or *C. liberica* var. *dewevrei*. This study aims to determine the morphological variations of Liberica coffee variant in Kalipuro Sub-district, Banyuwangi Regency, to provide essential basic data for future genetic confirmation and to support conservation strategies.

MATERIALS AND METHODS

Study Area

This study was conducted from August to October 2022 in Kalipuro Sub-district, Banyuwangi Regency, East Java, Indonesia (7° 43' - 8° 46' South Latitude and 113° 53' - 114° 38' East Longitude) (Banyuwangikab, 2022a) (Figure 1). Banyuwangi Regency is located in the eastern part of Java Island and is primarily an agricultural region, dominated by extensive wet and dry land farming (Hakim et al., 2015). It is bordered by the Java Sea, the Bali Strait, and the Indian Ocean, with a tropical climate possess two seasons: rainy and dry seasons (Banyuwangikab, 2022b).

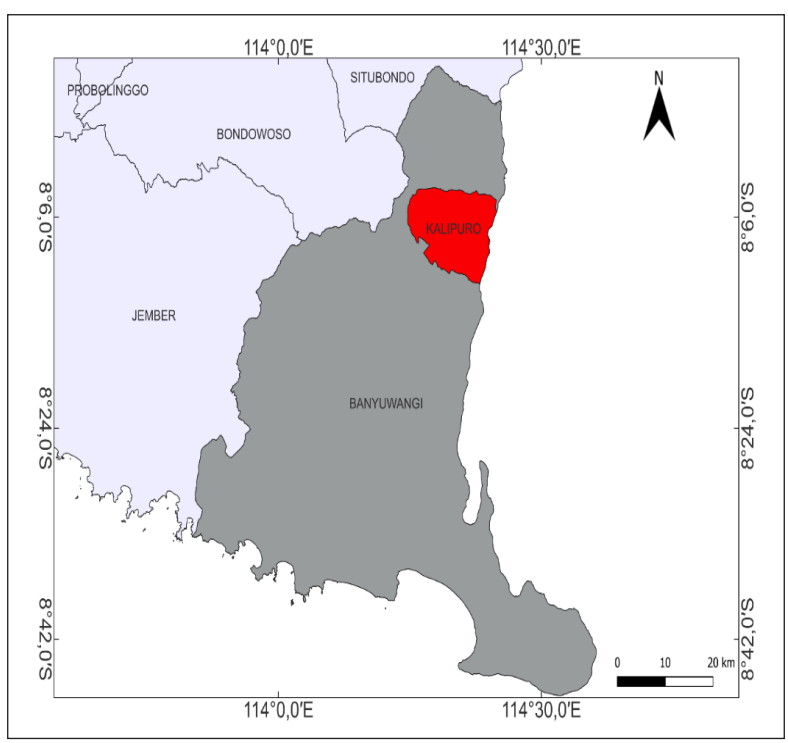

Figure 1. Location of the sampling site, Kalipuro Sub-district (Mukhoyyaroh et al., 2024)

Data Collection

Field studies were conducted to identify the reference morphological traits that would serve as the basis for classifying variants of Liberica coffee. The observed traits included qualitative and quantitative leaf characteristics (Ramadiana et al., 2018). Quantitative traits measured were trunk diameter; leaf length and width; petiole length; internode distance; fruit length and width, thickness, and weight; fruit flesh thickness; and the length, width, thickness, and weight of both hard-skinned beans and green beans. Leaf observations were conducted on mature leaves located more than three nodes from the terminal bud.

Leaf shape index (LL/LW) and leaf surface index (LLxLW) were calculated from leaf length (LL) and leaf width (LW) ratio. Measurement of length (L), width (W), and thickness (T) for fruit, green beans, and hard skin (HS) beans were used to calculate fruit volume (FLxFWxFT), fruit shape index (FL/FW), HS volume (HLxHWxHT), HS shape index (HL/HW), green bean volume (GBLxGBWxGBT), and green bean shape index (GBL/GBW). A hard skin bean (HS) refers to a coffee bean covered with a hard skin, while a green bean is a coffee bean with the hard skin removed. The morphometry illustration is shown in Figure 2. Quantitative measurements were conducted on five leaves and 100 fruits, beans, and HS beans per individual tree as replications.

The observed qualitative morphological traits included branching type, leaf shape; apex shape, leaf margin, stipule shape, leaf venation, ripe fruit colour, young leaf and fruit colour, and bean colour. The assessment method followed the Descriptors for Coffee (*Coffea* spp. and *Psilanthus* spp.) by the International Plant Genetic Resources Institute (IPGRI) (IPGRI, 1996; Ramadiana et al., 2018; Walyaro, 2006;). Qualitative trait observations for *C. liberica* were conducted on five mature leaves and 100 mature fruits and beans per individual tree as replications. Detailed trait descriptions and scoring guidelines are provided in Supplementary Table 1.

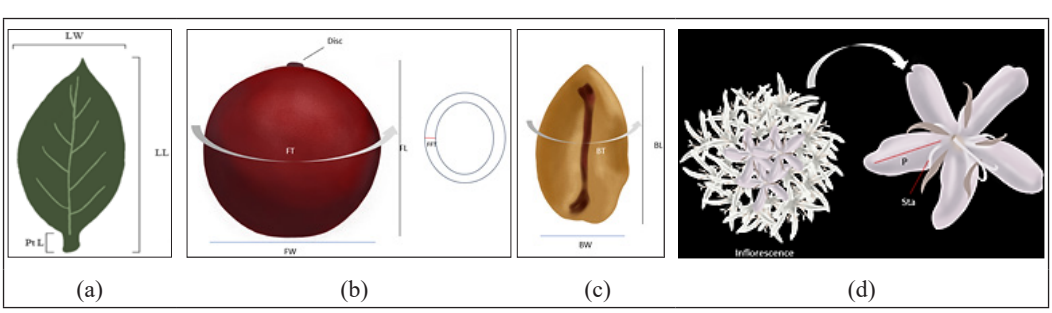

Figure 2. Morphological illustration of leaf measurement: a. leaf (LL: leaf length; LW: leaf width; Pt L; petiole length); b. fruit (FL: fruit length; FW: fruit width; FT: Fruit thickness; FFT: Fruit flesh thickness); c. bean (BL: bean length; BW: bean width; BT: bean thickness); d. Flower (P: petal; Sta: stamen)

Data Analysis

Measurement data for quantitative and qualitative morphological traits were tabulated, and statistical analysis was performed using standard deviation. Quantitative key characters were adapted from N'Diaye (2005) for *Coffea liberica* (Table 4), while the qualitative key character of bean shape was analysed separately (Table 2). Both qualitative and quantitative key characters were transformed into binary data for dendrogram construction using UPGMA algorithm based on Jaccard's similarity index, implemented in Paleontological Statistics (PAST) software.

Character states were subsequently analysed and interpreted manually to identify apomorphies (exclusive derived characters), following standard cladistic principles (Wiley & Liberman, 2011). Apomorphy can be divided into synapomorphies (shared by multiple accessions) and autapomorphies (unique to a single accession). Morphological traits were coded according to the observed variation, and character states were compared across all accessions. Autapomorphy was used to group individual accessions, while synapomorphies served only to describe cluster patterns. The identified apomorphic characters were then used to support the grouping patterns observed in the dendrogram and to provide diagnostic morphological traits for distinguishing among *Coffea liberica* accessions.

RESULTS

Morphological Leaf Variation of *Coffea liberica*

In plantations managed by local communities in Kalipuro Sub-district, coffee plants are predominantly *C. canephora* and *C. liberica*. *Coffea liberica* is locally known as "*kopi nangka"* or "jackfruit coffee" due to its distinctive jackfruit-like aroma and sour taste. Based on field exploration in coffee agroforestry plantations, four branching types of *C. liberica* were identified and labelled alphabetically as A, B, C, and D (Figure 3) to facilitate grouping. Type A is characterised by drooping branches with upward-curving tips. Type "A" is characterised by drooping branches with upward-curving tips. Type "B" is characterised by horizontal branches (90 degrees), type "C" by drooping (declinate) branches, and type "D" by upward-leaning branches at a 45° angle (Hickey & King, 2000; Tjitrosoepomo, 2011). Field exploration indicated that the branching type of *C. liberica* does not influence the morphological variation observed in the Kalipuro variant. Morphological trait variations were present across all branch types, with no specific traits associated exclusively with a particular branching type.

The leaf morphology of Liberica coffee, based on the qualitative traits, showed some variations, while other traits showed morphological similarity (Supplementary Table 2). Across the four branch types, the coffee plants are suspected to be a variant of *C. liberica* (*C. liberica* var. *liberica* and *C. liberica* var. *dewevrei*) that share certain leaf traits.

Distinguishing characteristics among accessions included variations in young leaf colour, mature leaf colour, leaf shape, apex leaf shape, and leaf margin (Supplementary Table 2). Young leaves from 17 accessions of *C. liberica* displayed a range of colours—reddish, brownish, and brownish green—with brownish being the most common (Figure 4). Meanwhile, mature leaf colours were predominantly dark green. Two accessions with the "D" branch type (D2, and D4) had green mature leaves, while all other “D” accessions, as well as all those from the "A", "B", and "C" types, had dark green mature leaves.

All the accessions of *C. liberica* had a similar elliptic leaf shape, although three accessions (A2, A5, and C3) had broader leaves, giving the elliptic shape a more rounded appearance (Figure 4). Leaf apex shape was either acuminate or acute. Only two accessions, A5 and C3, had an acute apex, both also showing rounded elliptic leaf shapes. Although A2 had a similar leaf shape, its apex was acuminate. All branch types exhibited leaves with either entire or undulate margins (Figure 4). Twelve of the 17 accessions had undulating margins, while the others had entire margins. All accessions had glossy leaf surfaces, triangular stipules, green petiole colour, and pinnate venation.

Quantitative leave traits indicated that all the *C. liberica* plants could be classified as *C. liberica* var. *dewevrei* based on key characteristics referenced in N'Diaye et al. (2005) (Table 4). The leaf surface index ranged from 313.79 to 721 cm^2, with an average of 541.93 cm^2 (Table 1), while the leaf shape index ranged from 1.69 to 2.45, with an average of 2.01. Petiole lengths ranged from 1.84 to 3.00 cm, affecting the shape and surface index. Another measured quantitative morphological trait was internode distance, which ranged from 5.30 cm to 10.58 cm. The smallest internode distance was found in the C3 accession, while the largest was found in the A5. Internode distance affects the productivity of Liberica coffee per individual tree, as it is related to the position of coffee fruit growth on the axillary internodes.

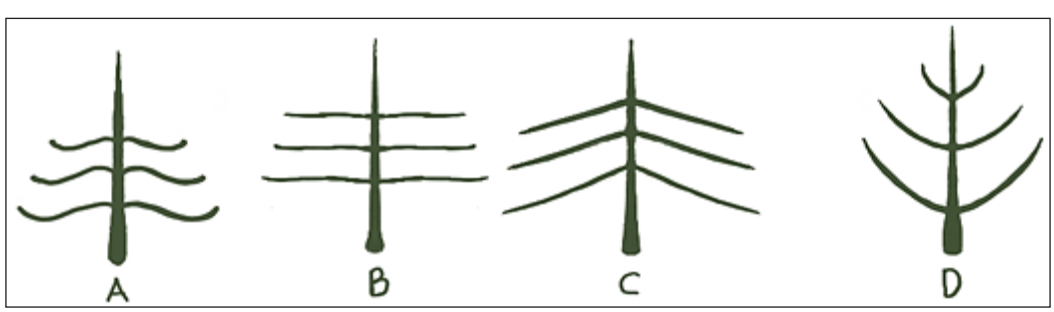

Figure 3. Illustration of branch types found in coffee plantations in Kalipuro Sub-district

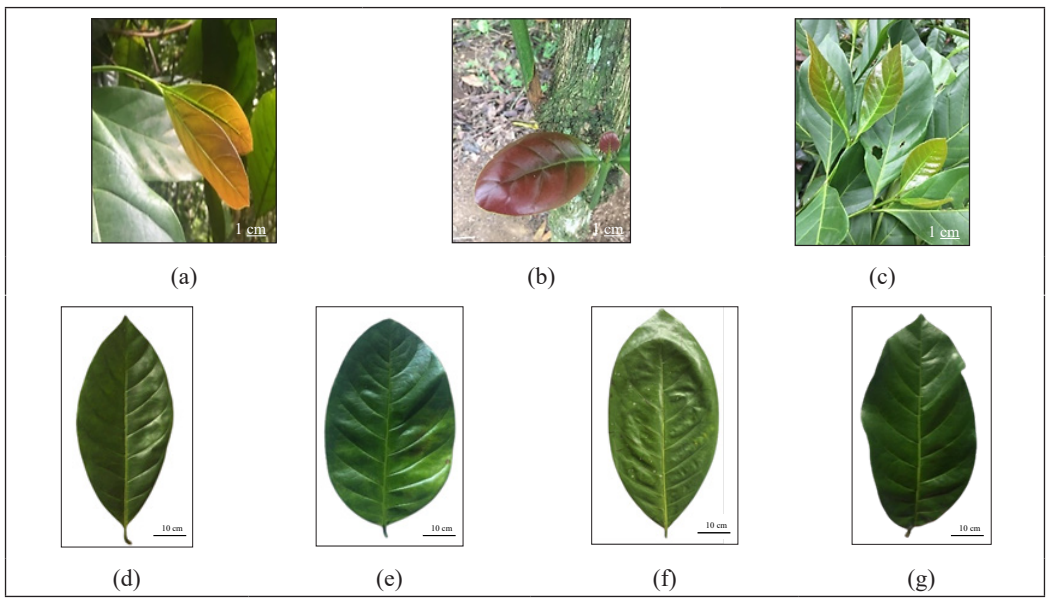

Figure 4. Coffea liberica vegetative traits: a. brownish young leaf colour; b. reddish young leaf colour; c. green-brownish young leaf colour; d. leaf shape elliptic with acuminate apex shape; e. elliptic tending to be rounded with an acute apex shape; f. mature leaf green colour with entire margin; g. mature leaf dark green colour with an undulate margin

Table 1
Quantitative morphology of leaves and internodes of C. liberica

Accession	Leaf Surface Index (cm^2)	Leaf Shape Index	Petiole Length (cm)	Internode Distance (cm)
A1	615.79±139.99	2.05±0.16	2.64±0.35	7.60±2.45
A2	625.65±134.75	1.79±0.06	2.54±0.15	9.95±3.72
A3	568.16±147.69	2.45±0.21	2.52±0.37	8.83±3.49
A4	467.84±123.59	1.87±0.24	1.84±0.21	10.24±3.40
A5	569.66±69.19	1.69±0.11	2.58±0.24	10.58±3.85
B1	612.8±66.73	1.95±0.09	2.16±0.27	8.00±3.50
B3	721±123.52	2.12±0.14	3.00±0.31	8.00±2.94
B5	485.69±112.11	2.07±0.10	1.66±0.42	6.04±1.86
C1	480.39±122.97	1.88±0.42	2.72±0.73	8.99±8.89
C2	432.13±99.22	2.01±0.23	2.08±0.29	6.69±2.70
C3	570.85±50	1.69±0.15	2.54±0.29	5.30±2.00
C4	313.79±34.23	2.15±0.20	1.88±0.39	6.13±2.56
C5	587.66±44.24	2.23±0.17	2.80 ± 0.27	6.59±3.69
D2	482.59±84.05	2.05±0.13	2.00±0.35	6.99±3.79
D3	606.47±174.86	2.28±0.24	2.80 ± 0.45	8.90±3.76
D4	592.62±23.16	2.07±0.18	1.92±0.19	8.04±2.84
D5	479.67±42.3	1.90±0.11	2.38±0.36	7.43±2.34
Average	**541.93±95.07**	**2.01±0.20**	**2.36±0.40**	7.90±1.53

Morphological Variations of *Coffea liberica* Fruit and Bean

Two variations in ripe fruit colour were observed among *C. liberica* accessions*:* red-orange (C2 and C4 Accessions) and red (the rest of accession) (Table 2; Figure 5). Two variations in bean colour were found in Liberica coffee, yellow and brown. Six of the 17 accessions (A3, B3, B5, C1, C2, and C3) had brown beans, while the others had yellow beans. However, this characteristic is not considered reliable for varietal differentiation, as it may change during moisture reduction and post-harvest processing. Liberica coffee variants also exhibited variations in fruit shape, including rounded and obovate forms. (Table 2; Figure 5). Notably, rounded fruits do not always produce a rounded bean shape, and an obovate fruit does not always yield an elliptic bean shape. For example, the B3 accession had an obovate fruit shape but a rounded bean. Similarly, some fruits with a rounded shape produced an elliptic bean shape. *Coffea liberica* var. *liberica* is typically associated with an elliptic bean shape, while *Coffea liberica* var. *dewevrei* is known for its rounded bean (Lagman, 2023; Sanchez et al., 2018). The *C. liberica* variety examined in this study display rounded and elliptical bean shapes, suggesting the possibility that these variants may be the hybrids of *liberica* and *dewevrei* types. However, previous research indicates that crossing *Coffea liberica* var. *liberica* with *Coffea liberica* var. *dewevrei* yields fertile offspring. Therefore, the variation in bean shape observed in *C. liberica* from Kalipuro is likely the result of hybridisation between the two varieties.

One of the notable traits in coffee plants is the fruit disc—the point of flower attachment, which varies among coffee plants. The disc shape found in the Liberica coffee variant in the Kalipuro Sub-district included: (1) small diameter with small protrusions, (2) large diameter with small protrusions, (3) large diameter with very prominent protrusions, (4) small diameter that was flat or not prominent, and (5) large diameter that was flat or not prominent (Table 2). The C4 and D5 accessions exhibited discs with large diameters and small protrusions, as well as large, very prominent discs. In contrast, most other accessions present fruit discs with small diameters and small protrusions. Accession A3 was found to have a distinctly large, very prominent disc. Notably, a large disc diameter does not always correspond with prominent protrusions, as seen in accession B3.

Table 2
Qualitative morphology of fruit and beans

Accession	Fruit Colour	Bean Colour	Fruit Shape	Bean Shape	Disc Shape
A1	Red	Yellow	rounded	elliptic	Small diameter, small protrusion
A2	Red	Yellow	obovate	elliptic	Small diameter, small protrusion
A3	Red	Brown	rounded	rounded	Large diameter very prominent
A4	Red	Yellow	rounded	elliptic	Small diameter, small protrusion
A5	Red	Yellow	obovate	elliptic	Small diameter, flat; small diameter, small protrusion

Table 2 (*continued*)

Accession	Fruit Colour	Bean Colour	Fruit Shape	Bean Shape	Disc Shape
B1	Red	Yellow	obovate	elliptic	Small diameter, flat; small diameter, small protrusion
B3	Red	Brown	obovate	rounded	Large diameter, small protrusion; large diameter, very prominent
B5	Red	Brown	rounded	rounded	Small diameter, small protrusion
C1	Red	Brown	rounded	elliptic	Small diameter, small protrusion
C2	Red-orange	Brown	rounded	rounded	Small diameter, small protrusion
C3	Red	Brown	rounded	elliptic	Small diameter, small protrusion
C4	Red-orange	Yellow	obovate	elliptic	Large diameter, very prominent; large diameter, small protrusion
C5	Red	Yellow	obovate	elliptic	Small diameter, very prominent; large diameter, very prominent
D2	Red	Yellow	rounded	rounded	Small diameter, small protrusion
D3	Red	Yellow	rounded	elliptic	Small diameter, small protrusion
D4	Red	Yellow	rounded	rounded	Small diameter, small protrusion
D5	Red	Yellow	rounded	rounded	Large diameter, small protrusion; large diameter, very prominent

The fruit volume of *C. liberica* ranged from 2.08 to 4.17 cm^3, with an average of 2.78 cm^3 (Table 3). Compared to a previous study (N'Diaye et al*.,* 2005) conducted in native populations in Africa, the Liberica coffee cherries from the Kalipuro Sub-district population can be grouped as suspected *Coffea liberica* var. *dewevrei*. The fruit shape index ranged from 0.16-1.52, with an average of 1.08. Quantitative measurements also showed variation in bean volume: hard skin (HS) bean volume ranged from 0.44 to 0.80 cm^3 (average 0.60 cm^3), while green bean volume ranged from 0.07 to 0.68 cm^3 (average 0.41 cm^3). The HS shape index ranged from 1.28 to 1.81 (average o1.44), and the green bean shape index ranged from 1.30 to 2.06 (average 1.51). The Liberica coffee variant is known to have thicker fruit flesh compared to other commercial coffee plants. This study revealed that fruit flesh thickness ranged from 0.71 to 1.42 mm. The thinnest was found in accession B5 (0.71 ± 0.30 mm), while the thickest was observed in accession A2 (1.42 ± 0.41 mm).

Morphological Variation of *Coffea liberica* Flower

Coffee flowers are arranged in the axils between internodes, with white petals and attached stamens. Generally, each flower has five petals and stamens, though some flowers have six, and rarely eight. However, Liberica coffee in this study showed 5 to 6 petals and stamens (Figure 5), with the number of petals equal to the number of stamens in all flowers observed.

Table 3
Quantitative morphology of fruit and beans

Accession	Fruit Weight (g)	HS Weight	Green Bean Weight	Fruit Flesh Thickness (mm)	Fruit Volume (cm^3)	Fruit Shape Index	HS Volume (cm^3)	HS Shape Index	Green Bean Volume (cm^3)	Green Bean Shape Index
A1	2.50±0.40	0.35±0.05	0.26±0.04	0.92±0.24	3.41±0.64	1.10±0.09	0.67±0.09	1.56±0.11	0.37±1.55	1.55±0.12
A2	1.53±0.24	0.35±0.05	3.01±0.04	1.42±0.41	2.22±0.33	1.12±0.07	0.51±0.08	1.44±0.10	0.07±1.39	1.39±0.08
A3	1.41±0.36	0.65±3.27	0.25±0.03	0.86±0.94	2.61±0.57	1.03±0.08	0.45±0.06	1.31±0.06	0.37±1.30	1.30±0.08
A4	1.35±0.28	0.31±0.03	0.24±0.03	0.73±0.21	2.24±0.50	1.15±0.09	0.44±0.07	1.41±0.14	0.38±1.42	1.42±0.07
A5	1.84±0.32	0.40±0.05	0.30±0.03	0.76±0.16	2.85±0.56	1.12±0.10	0.62±0.08	1.42±0.10	0.27±1.46	1.46±0.16
B1	1.59±0.34	0.37±0.06	0.27±0.05	0.90±0.29	2.20±0.56	1.08±0.11	0.56±0.09	1.34±0.08	0.43±1.35	1.35±0.15
B3	2.31±0.60	0.59±0.06	0.45±0.05	0.86±0.18	3.32±0.96	1.12±0.11	0.84±0.09	1.37±0.09	0.68±1.36	1.36±0.07
B5	2.02±0.23	0.44±0.04	0.30±0.03	0.71±0.30	2.56±0.43	1.11±0.09	0.59±0.06	1.52±0.09	0.46±1.51	1.51±0.16
C1	2.74±0.46	0.52±0.07	0.38±0.05	1.06±0.19	4.17±0.78	1.13±0.09	0.61±0.07	1.49±0.11	0.60±1.49	1.49±0.10
C2	2.64±0.46	0.53±0.08	0.39±0.05	0.85±0.23	3.51±0.72	0.99±0.10	0.80±0.11	1.51±0.10	0.61±1.36	1.36±0.08
C3	1.96±0.29	0.42±0.06	0.33±0.05	0.94±0.21	2.78±0.42	1.22±0.10	0.74±0.09	1.38±0.07	0.49±1.57	1.57±0.12
C4	1.27±0.27	0.33±0.04	0.26±0.03	0.92±0.26	2.08±0.60	1.52±0.16	0.49±0.99	1.31±0.21	0.38±1.88	1.88±0.21
C5	1.68±0.48	0.41±0.04	0.32±0.03	0.95±0.29	2.53±0.92	1.35±0.13	0.59±0.13	1.81±0.27	0.50±1.81	1.81±0.12
D2	1.72±0.47	0.41±0.07	0.33±0.06	0.94±0.16	2.67±0.77	0.16±0.01	0.48±0.06	1.35±0.11	0.07±1.46	1.46±0.09
D3	2.04±0.33	0.39±0.05	0.30±0.05	0.79±0.23	2.40±0.69	1.07±0.07	0.60±0.10	1.47±0.08	0.47±1.50	1.50±0.14
D4	2.12±0.34	0.41±0.07	0.33±0.06	0.87±0.19	2.87±0.47	0.97±0.05	0.57±0.10	1.54±0.11	0.51±1.25	1.25±0.07
D5	1.52±0.37	0.39±0.04	0.30±0.04	0.76±0.19	2.92±0.53	1.08±0.06	0.61±0.11	1.28±0.06	0.28±2.06	2.06±0.26
Average	**1.90 ±0.45**	**0.43±0.09**	**0.44±0.6**	**0.90 ±0.16**	**2.78±0.55**	**1.08±0.27**	**0.60±0.11**	**1.44±0.13**	**0.41±0.17**	1.51±0.22

Note. HS=hard skin

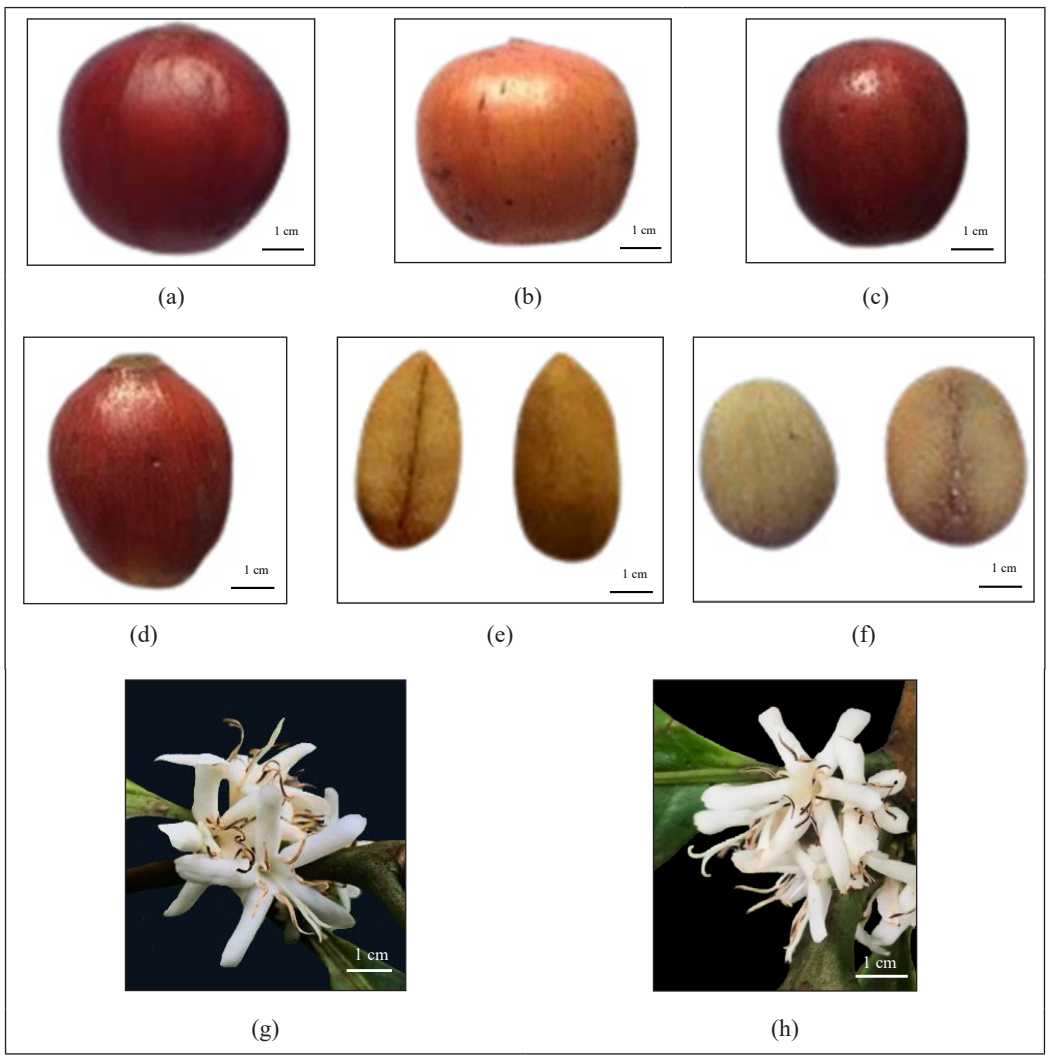

Figure 5. Coffea liberica generative traits: a. red fruit colour; b. red-orange fruit colour; c. rounded fruit shape; d. obovate fruit shape; e. yellow-elliptic bean; f. brown-rounded bean; g. flower with five petals; h. flower with six petals

The number of flowers per inflorescence ranged from 9 to 26 flowers, and inflorescent stalk lengths across the 17 *C. liberica* coffee accessions ranged from 0.30 cm to 0.60 cm, indicating variability. However, these traits could not serve as distinguishing characteristics among Liberica variants. Based on comparison with the findings of N'Diaye et al. (2005), the Liberica coffee variant from Kalipuro Sub-district is suspected to be var. *dewevrei,* based on the number of flowers/inflorescence. However, the number of flowers/inflorescence in the Liberica variants from Kalipuro Sub-district was higher than that reported in the previous study.

Based on the interpretation of all traits, the Liberica coffee variants can be grouped based on key characters that significantly distinguish them from those reported in the previous study (Table 4). Additionally, bean shapes (Table 2) serve as an important trait for distinguishing Liberica variants, as illustrated in the dendrogram (Figure 6). Using Jaccard's similarity coefficient of 0.7, the 17 *C. liberica* accessions from Kalipuro Sub-district were closely similar to *C. liberica* var. *dewevrei* and known to be distinct from *C. liberica* var. *liberica*.

Apomorphy is an evolutionary novelty that arises in a particular lineage, which can be either synapomorphies (shared among multiple accessions) or autapomorphies (unique to a single accession), providing valuable information for determining phylogenetic relationships (Choudhuri, 2014; Wiley & Lieberman, 2011). Autapomorphy refers to a unique trait found only in a single taxon that does not appear in another taxon, serving as a distinctive marker for taxonomic identification. Using this framework, accession C1 formed a separate subclade due to its larger fruit volume, representing an autapomorphy. This trait supports the distinct position of C1 in the dendrogram. Meanwhile, shared derived characters identified in the *C. liberica* var. *dewevrei* clusters include a leaf surface index > 200 cm², a leaf shape index ≤ 2.2, and more than three flowers per inflorescence. These traits help define the cluster but are not used for differentiating individual accessions.

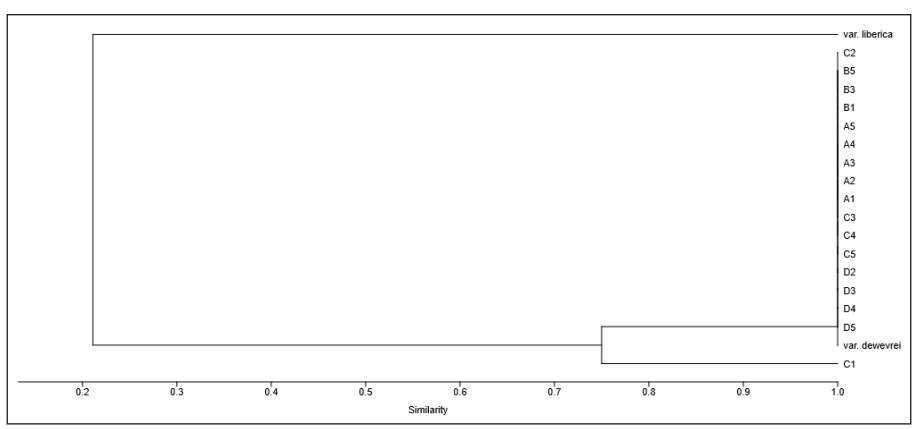

Figure 6. Coffea liberica dendrogram based on morphological traits using Jaccard's similarity

DISCUSSION

The variations in qualitative and quantitative traits are influenced by several factors, including the environment, genotypes, and possible interactions between the two factors (Martono, 2017). The relationships among coffee morphological traits are also influenced by several agronomic trait expressions, which produce heterogeneous effects when combined with inter- or intra-species occurrences (Kusolwa et al., 2019).

Table 4
Morphological comparison of C. liberica *found in Kalipuro Sub-district in comparison to a previous study*

	***C. liberica* variants**																	***C. liberica* Reference (N'Diaye et al., 2005)**	
Accession	**A1**	**A2**	**A3**	**A4**	**A5**	**B1**	**B3**	**B5**	**C1**	**C2**	**C3**	**C4**	**C5**	**D2**	**D3**	**D4**	**D5**	***var. liberica***	***var. dewevrei***
Leaf surface index (cm^2)	615.79 ± 139.99	625.65 ± 134.75	568.16 ± 147.69	467.84 ± 123.59	569.66 ± 69.19	612.8 ± 66.73	721 ± 123.52	485.69 ± 112.11	480.39 ± 122.97	432.13 ± 99.22	570.85 ± 50	313.79 ± 34.23	587.66 ± 44.24	482.59 ± 84.05	606.47 ± 174.86	592.62 ± 23.16	479.67 ± 42.3	131.20 (66.00-226.20)	218.20 (111.60-307.40)
Leaf shape index	2.05 ± 0.16	1.79 ± 0.06	2.45 ± 0.21	1.87 ± 0.24	1.69 ± 0.11	1.95 ± 0.09	2.12 ± 0.14	2.07 ± 0.1	1.88 ± 0.42	2.01 ± 0.23	1.69 ± 0.15	2.15 ± 0.2	2.23 ± 0.17	2.05 ± 0.13	2.28 ± 0.24	2.07 ± 0.18	1.9 ± 0.11	2.40 (1.90-2.80)	2.20 (1.70-2.70)
Number of Flowers/ inflorescences	16	13.60	13.80	8.60	10.60	17.60	16.20	20.80	22.60	15.20	16.60	9.20	9.80	13.60	26	19.60	20.40	2.90 (1.20-4.60)	3.30 (2.20-6.90)
Fruit volume (cm^3)	3.41 ± 0.64	2.22 ± 0.33	2.61 ± 0.57	2.24 ± 0.5	2.85 ± 0.56	2.2 ± 0.56	3.32 ± 0.96	2.56 ± 0.43	4.17 ± 0.78	3.51 ± 0.72	2.78 ± 0.42	2.08 ± 0.6	2.53 ± 0.92	2.67 ± 0.77	2.40 ± 0.69	2.87 ± 0.47	2.92 ± 0.53	4.00 (2.00-6.60)	2.90 (1.10-4.30)

Source: N'Diaye et al. (2005)

Adaptation of plants to environmental conditions influences the insertion angle between the trunk and the branch, leading to different branch growth directions. Coffee plantations with agroforestry systems generally apply multi-strata plants that serve as shade plants, affecting the light intensity captured by plants. Adaptation to shade or tree canopies is exemplified by the unique type "A" branch. Plants grown in shade-intolerant conditions will branch out towards light to increase light absorption by optimising the photosynthesis rate, as light is an important factor in photosynthesis (Ruberti et al., 2012). The direction of plant growth will generally lead to a source of sunlight (phototropism). Through phototropic responses, plants can reorient their growth direction toward or away from the light source to optimise photosynthetic light capture in the aerial portion (Liscum et al., 2014).

The leaf colour variations are also influenced by the canopy shade, affecting the light absorption efficiency by leaves (Yustiningsih, 2019). As shading increases, leaf brightness decreases. This change in leaf colour correlated with an increase in chlorophyll concentration (Brand, 1997; Kurniawan et al., 2021). Meanwhile, in the lower plants, the leaf colour of plants grown under intense light showed the darkest leaf colour compared to those under intense canopy (Rezai et al., 2018). Under low light intensity, chloroplasts concentrate on the sides of the cell walls, farthest from the light, causing leaves in shaded conditions to appear greener (Kisman et al., 2007). In such conditions, plants showed shade tolerance and avoidance strategies. Shade tolerance responses include increased chlorophyll content, specific leaf area, and photosystem II:I ratio, along with a decreased chlorophyll a:b ratio (Charrier, 2021; Wu et al., 2017).

The differences in the quantitative morphological traits in Kalipuro Sub-district differs from the previous study by N'Diaye et al. (2005) regarding the results of measurements on the original population of the Liberica coffee variants. Regarding the leaf size of *C. liberica*, the adaptive response of plants to specific conditions leads the plants to create smaller and narrower leaves to reduce evaporation (Morais et al., 2004). A previous study by Pompelli et al. (2012) also stated that Arabica coffee plants with full sun conditions have smaller leaves, enabling an increase in the effectiveness of cooling under conditions of high light intensity. Regarding leaf morphological variations, previous studies also mentioned that Liberica coffee variants have a round, elongated leaf shape (Santos & Cao, 2020; Udarno & Setiyono, 2015). Leaf margin in this research aligns with previous research (Baltazar & Buot, 2019), revealing that both variants have entire leaf margins and undulate features. Variations in leaf shape are caused by genetic control, while environmental factors are crucial for the final adjustment of the leaf shape (Dkhar & Pareek, 2014).

Shaded conditions also reduce temperature, which slows down the fruit ripening process. In addition, fertilisation factors affect bean size, weight, and organoleptic quality through the accumulation of fats and carbohydrates (Bote & Vos, 2017). Natural adaptive strategies influence fruit colour, shape, and size variation. Fruit shape is correlated with

the direction, period of cell division, and expansion, influencing fruit shape and ovary size (Wang et al., 2022). Genetic regulation also controls fruit shape and represents a quantitative trait involving complex interactions among multiple genes and environmental factors. The environmental factors influencing the fruit include temperature, light, and humidity (Liu et al., 2024). The coffee fruit and bean present various morphological shapes among coffee species and varieties. *Coffea liberica* var. *liberica* has an elliptic shape and is longer and wider than *C.liberica* var. *dewevrei* (Sanchez et al., 2018). *Coffea liberica* var. *dewevrei* in the Kalipuro Sub-District has a smaller bean size compared to *C. liberica* var. *liberica*, which aligns with the previous study by Lagman (2023), who stated that *C. liberica* var. *dewevrei* has a smaller and less elongated bean. The coffee yield is also influenced by the number of primary branches and internode distances in coffee plants, affecting coffee productivity (Martono, 2017).

The number of flowers per inflorescence determines the maximum number of fruits. However, a successful fruit set depends on effective pollination and environmental conditions (Centeno-Alvarado et al., 2024; Klein et al., 2003). The environmental factors affecting flowering are rainfall, water supply (Charrier & Berthaud, 1985), and photoperiod (Unigarro et al., 2023). High rainfall intensity caused coffee flowers to fall off, potentially decreasing the number of flowers per inflorescence. High rainfall intensity interferes with the pollen transfer process, thus hampering the reproductive process of flowering plants (Lawson & Rands, 2019). Genetic factors also influence plant flowering. The *MIKC1* gene is suspected to regulate the number of inflorescences by increasing inflorescences per branch and promoting flower production (Xu et al., 2024). Flower quantification in coffee plants is influenced by photoperiod in the equator region. Water supply and temperature also significantly influence flowering and fruit ripening. The coffee flowers develop from serial buds and are grouped in inflorescences located in the axils of plagiotropic branches. Floral structure can vary among genotypes within the same species (Silva et al., 2024).

The morphological variations in coffee, especially Liberica coffee, are strongly affected by environmental factors. The morphological variation of the Liberica coffee in the Kalipuro Sub-district can be clustered as *C. liberica* var. *dewevrei*, based on key characters, referred to the original African population of Liberica coffee described by N'Diaye et al. (2005), and bean shape as an important character. The clustered *C. liberica* var. *dewevrei* from Kalipuro Sub-district reveals a potential variation from its group (C1), based on fruit volume as an autapomorphy. However, this study cannot state that C1 is a new variant of *C. liberica* var. *dewevrei* from Kalipuro Sub-district due to the small sample population size. Further research with a larger sample population is required to confirm the findings. Inconsistencies in several qualitative morphological traits were considered insignificant for comparing variants, highlighting the need for an in-depth molecular study to confirm the taxonomic position of *C. liberica*. The high similarity of *C. liberica* morphological

traits can be influenced by the origin of the planted seeds. In addition, environmental factors are believed to strongly influence changes in plant morphology as adaptations to surrounding conditions; consequently, such morphological changes are often considered to be plastic in nature. Considering the introduction of Liberica coffee in Java Island, including on the east slope of Ijen, which occurred a hundred years ago, environmental influences may lead to variations and speciation, considering the evolution of *C. liberica*. Therefore, a comprehensive study of molecular variations must be conducted to confirm the morphological variations.

CONCLUSION

The study revealed that, morphologically, Liberica coffee in Kalipuro Sub-district is more closely related to *C. liberica* var. *dewevrei* compared to *C. liberica* var. *liberica*. Morphological analyses included measurement of quantitative traits (leaf surface and shape index, number of flowers per inflorescence, fruit volume) and qualitative traits (bean shape) followed by coding of character states into binary data, and dendrogram construction using Jaccard's similarity index and UPGMA clustering. Apomorphy, particularly autapomorphy, was applied to identify unique traits among individual accessions. Among all traits, fruit volume showed as the most distinctive characteristic for differentiating accessions, allowing C1 to be recognised as a potential new variant of *Coffea liberica* var. *dewevrei*; however, further in-depth study is required to conduct a valid and reliable identification. Due to the presence of numerous shared traits, differentiating these varieties remains complex, and additional genetic studies are necessary to confirm the taxonomic status of Liberica coffee.

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SUPPLEMENTARY DATA

Table S1
Assessment method for Coffea

Plant Organ	Descriptor	Reference	Assessment Method and Scale
Stem	Main branch direction	(IPGRI, 1996)	Observed on main stem; 1 = drooping, 2 = horizontal or spreading, 3 = semi-erect, 4 = other
	Stem diameter	(IPGRI, 1996)	Measured at 5 cm above the soil surface in seedlings and cuttings and 10 cm above the graft union in grafted trees by estimating the age of the tree
Leaf	Young leaf colour	(IPGRI, 1996)	1 = greenish, 2 = green, 3 = brownish, 4 = reddish, 5 = bronze (metallic brown), 6 = other
	Mature leaf colour	(IPGRI, 1996)	Observed on leaves located more than three nodes from the terminal bud; 1 = dark green, 2 = green, 3 = other
	Leaf shape	(IPGRI, 1996)	1 = obovate, 2 = ovate, 3 = elliptic, 4 = lanceolate, 5 = elliptic tending to round
	Leaf tip shape	(IPGRI, 1996)	1 = round, 2 = obtuse, 3 = acute, 4 = acuminate, 5 = apiculate, spatulate, 6 = other
	Stipule shape	(IPGRI, 1996)	1 = round, 2 = ovate, 3 = triangular, 4 = deltate, 5 = trapeziform, 6 = other
	Leaf length	(IPGRI, 1996)	Average of 5 mature leaves (observed on leaves located more than 3 nodes from the terminal bud), measured from petiole to leaf tip
	Leaf width	(IPGRI, 1996)	Average of 5 mature leaves (observed on leaves located more than 3 nodes from the terminal bud), measured at the widest part of the leaf
	Petiole length	(IPGRI, 1996)	Average 5 leaves, measured from the base to the insertion of the leaf blade
	Petal colour	(IPGRI, 1996)	1 = green, 2 = dark brown, 3 = other
Flower	Number of flowers per axil/ inflorescence	(IPGRI, 1996; Walyaro, 2006)	An average of 10 axils, randomly selected from different nodes
	Long inflorescent stalks	(IPGRI, 1996)	An average of 5 inflorescences, randomly selected from different nodes
	Number of petals per flower	(IPGRI, 1996)	An average of 5 flowers, randomly selected from different nodes
	Number of stamens per flower	(IPGRI, 1996)	An average of 5 flowers, randomly selected from different nodes

Table S1 (*continued*)

Plant Organ	Descriptor	Reference	Assessment Method and Scale
Fruit	Fruit colour	(IPGRI, 1996; Walyaro, 2006)	Observed on ripe fruit; 1 = yellow, 2 = yellow-orange, 3 = orange, 4 = reddish-orange, 5 = red, 6 = purplish-red, 7 = purple, 8 = purple-violet, 9 = violet, 10 = black, 11 = other
	Fruit shape	(IPGRI, 1996; Walyaro, 2006)	Average of 5 normally ripe fruit; 1 = round, 2 = obovate, 3 = ovate, 4 = elliptic, 5 = oblong, 6 = other
	Fruit disc shape	(IPGRI, 1996)	Observed at the tip of the cherry; 1 = absent, 2 = present but not prominent, 3 = prominent (cylindrical), 4 = beaked (resembling the shape of a bottleneck)
	Fruit length	(IPGRI, 1996; Walyaro, 2006)	Average of 100 mature fruit, measured at the longest part
	Fruit width	(IPGRI, 1996; Walyaro, 2006)	Average of 100 mature fruit, measured at the widest part
	Fruit thickness	(IPGRI, 1996)	Average of 100 mature fruit, measured at the thickest part
	Flesh thickness	(IPGRI, 1996)	Observation with a scale; 3 = thin, 5 = medium, 7 = thick
Bean	Bean length	(IPGRI, 1996)	Average of 100 mature normal beans, measured at the longest part
	Bean width	(IPGRI, 1996)	Average of 100 mature normal beans, measured at the widest part
	Bean thickness	(IPGRI, 1996)	Average of 100 mature normal beans, measured at the thickest part
	Bean colour	(IPGRI, 1996; Walyaro, 2006)	1 = yellow, 2 = purplish brown, 3 = brown
	Bean shape	(IPGRI, 1996; Walyaro, 2006)	1 = round, 2 = obovate, 3 = ovate, 4 = elliptic, 5 = oblong, 6 = other

Table S2
Qualitative leaf morphology of C. liberica

Accession	Young Leaf Colour	Mature Leaf Colour	Leaf Shape	Leaf Margin	Apex Shape	Stipule Shape	Petiole Colour	Leaf Surface	Venation
A1	reddish	dark green	elliptic	undulate	acuminate	triangular	green	glossy	pinnate
A2	brownish	dark green	elliptic (slightly rounded)	entire	acuminate	triangular	green	glossy	pinnate
A3	brownish	dark green	elliptic	undulate	acuminate	triangular	green	glossy	pinnate
A4	brownish	dark green	elliptic	undulate	acuminate	triangular	green	glossy	pinnate
A5	brownish	dark green	elliptic (slightly rounded)	undulate	acute	triangular	green	glossy	pinnate
B1	brownish	dark green	elliptic	undulate	acuminate	triangular	green	glossy	pinnate
B3	brownish	dark green	elliptic	undulate	acuminate	triangular	green	glossy	pinnate
B5	reddish	dark green	elliptic	undulate	acuminate	triangular	green	glossy	pinnate
C1	brownish green	dark green	elliptic	undulate	acuminate	triangular	green	glossy	pinnate
C2	brownish green	dark green	elliptic	entire	acuminate	triangular	green	glossy	pinnate
C3	reddish	dark green	elliptic (slightly rounded)	entire	acute	triangular	green	glossy	pinnate
C4	brownish	dark green	elliptic	undulate	acuminate	triangular	green	glossy	pinnate
C5	reddish	dark green	elliptic	undulate	acuminate	triangular	green	glossy	pinnate
D2	brownish	green	elliptic	entire	acuminate	triangular	green	glossy	pinnate
D3	reddish	dark green	elliptic	entire	acuminate	triangular	green	glossy	pinnate
D4	reddish	green	elliptic	undulate	acuminate	triangular	green	glossy	pinnate
D5	reddish	dark green	elliptic	undulate	acuminate	triangular	green	glossy	pinnate

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Analogue Rice from *Amorphophallus muelleri* and Potato with Enhanced Nutritional Properties: Lower Glycemic Index and Higher Fibre Content

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ABSTRACT

In the year 2024, the world population is expected to reach 8.2 billion. At an estimated annual growth rate of 0.8%, the United Nations predicts that it will reach 9.7 billion by 2050 and 10.4 billion in the 2080s. Further, according to the World Food Programme, as many as 309 million people are facing chronic hunger. Sadly, the number of acutely hungry people and malnutrition continues to increase. Inline with the second and third goals of the 2030 Agenda for Sustainable Development, to end hunger, achieve food security, ensure healthy lives and promote well-being, this paper deals with an initiative to find the production of analogue rice, with a new composition of ingredients. The materials used are Indonesian konjac, potatoes, and several auxiliary ingredients such as glyceryl monostearate (GSM), sago, and water. The methods used were weighing, mixing, heating, and moulding using an extruder. Its objectives are to determine the optimum composition of the ingredients used and to assess its nutritional characteristics. More importantly, the analogue rice produced using this formula possessed the nutritional characteristics of healthy food. Additionally, the easy availability of the ingredients has the potential to contribute

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to alleviating the food affordability and availability crises. The conclusion on the best compositions was Indonesia konjac (39.50 %), *S. tuberosum* (19.50 %), Sago (9.50 %, Glyceryl Monostearate (GSM) (2.00 %), and water (29.50 %) (formula IV).

Keywords: Analogue rice, food security, Indonesian konjac, nutrition, *Solanum tuberosum*

INTRODUCTION

Human survival is one of the principal concerns of the 2030 Agenda for Sustainable Development Goals (SDGs). The first goal of this agenda is to end hunger, achieve food security, improve nutrition and promote sustainable agriculture. The next is to ensure healthy lives and promote well-being for all ages. However, according to the World Food Programme (WFP), unfortunately, the number of acutely hungry people, and thus malnutrition, continues to increase (FSIN, 2024). Almost 4% of the world's population, in 71 countries, is facing chronic hunger. Among them, 12% face emergency levels of hunger and around 0.5% are in the grips of catastrophic hunger (FSIN, 2024). More specifically, according to the United Nations Children's Fund (UNICEF), World Health Organisation (WHO), and World Bank (2023), an estimated 149 million children under the age of 5 were suffering from stunting. Further, currently, nearly half of the deaths among children under 5 years of age are linked to undernutrition, and another 190 million are living with thinness.

It is in the spirit of contributing towards ending hunger, achieving food security, improving nutrition, and promoting sustainable agriculture that this research was conducted, and its results were publicised so that authorities around the globe could take necessary actions. This topic is not new, as can be seen in the study by (Tie et al., 2008), where scientists and researchers tried to produce analogue rice (AR in brief) using plant tubers and some special additional ingredients while keeping the nutritional content as similar as possible to that of paddy rice. AR is a form of artificial rice made from various kinds of flour as a substitute for natural rice. Thus, its production process needs to be streamlined to result in characteristics similar to paddy rice, with carbohydrate content similar to or in excess of that in natural rice and sufficient nutritional content, such as protein, starch, and sugars (Sumardiono et al., 2021).

In this study, the Indonesian konjac (IK) tuber was chosen as the principal ingredient, the reason being that the konjac tuber has multiple benefits and has been used as raw material in various industries such as the food and beverage industry, pharmaceutical industry, and cosmetics industry (Widjanarko et al., 2022). The unique characteristics of konjac have also been frequently discussed in many literatures, such as Dwiyono and Djauhari (2019), Pan et al. (2015), and Thanh et al. (2013). More specifically, Dwiyono and Djauhari (2019) mentioned that IK flour contains 6.16% protein, 10.14% crude fibre,

and 35.92% glucomannan, which shows its potential for developing healthy artificial food. In relation to this, Harijati et al. (2018) mentioned that IK can be used to control obesity and diabetes. It is also a wild plant which can be easily cultivated across Indonesia and Southeast Asia (Wahidah et al., 2021).

Besides IK, additional carbohydrate sources are needed. According to Santoso et al. (2018) and Hasbullah et al. 2023, potential options include cassava, corn, maize, mocap, sago, sorghum, and soybeans. However, this study utilised potato as it offers a high carbohydrate content (Obong & Parmentier, 2009) and is readily available in Indonesia. IK and potato are good ingredients, but are insufficient to produce high-quality AR, as AR grains need to be solid, resistant to cracking, and non-stick to one another. Sago and glycerol monostearate (GMS) were the additional ingredients to resolve these issues. Finally, water was added as the fifth ingredient to keep the production machine safe from overheating. Thus, IK flour, potato flour, sago starch, GMS, and water were the five ingredients used to produce AR in this study. Although the use of these ingredients is not novel (Tie et al., 2008), determining the optimal composition or percentage of each ingredient to achieve the best results has remained challenging.

Therefore, the present study was carried out: 1. to investigate the different composition of ingredient formulations on the properties of AR and 2. to develop an AR porridge product with a palatable texture and pleasant flavour by adding konjac glucomannan.

MATERIALS AND METHODS

Materials Used

The main raw materials used in this study are porang tubers obtained from porang farmers in Klangon Village, Saradan Subdistrict, Madiun Regency, East Java Province, and potatoes of the "Granola" variety obtained from potato farmers in Dieng Village, Wonosobo Regency, Central Java Province. Sago flour was purchased from the Bogor market, West Java, Indonesia. Porang, potato tubers as the primary ingredient, and sago, GMS, and water as additional components; all of which were sourced from local vendors in Bogor market west Java, Indonesia. Chemicals such as sulphuric acid 98% Merck Germany, potassium permanganate 99% Riedel-de Haen, sodium malonate dibasic 98% Merck Germany, hydrochloric acid 98% Merck Germany, sodium chloride 98.5% Himedia, oxalic acid 98% Merck Germany, sodium sulfate 99% Merck German, aquadest, sodium hydroxide 99% Nepa-Smart, hexane 99% Merck Germany, and ethanol 98% Merck Germany.

The laboratory utilised was equipped with standard tools and instruments, as suggested in the literature, the grinder hammer mill, grinder disc mill, mixer, a double screw extruder, Fourier Transform Infrared (FT-IR) analyser, and a digital optical microscope (DOM) from Bogor Agriculture University.

Methods of Data Collection and Analysis

To determine the best composition formula, it was first written as X% of IK flour, Y% of potato flour, Z% of sago starch, V% of GMS, and W% of water. The principal problem addressed in this research is: "How to find the optimal values of X, Y, Z, V and W?" To answer this question, four composition formulas were considered, and laboratory experiments were conducted for each formula.

The optimal formula was the one which resulted in a product with quality characteristics most similar to those of natural rice. Here, the word "similar" relates to the levels of moisture, ash, fat, protein, carbohydrates, fibre, calcium oxalate, bond profile (functional group), and micro-structural profile. For this purpose, this study encompassed seven analyses:

1. Formulation analysis to make sure that the product possessed the flavour of natural rice.
2. Proximate analysis to compare its chemical composition with that of natural rice.
3. Physical characteristics analysis to examine its significant quality characteristics.
4. Rapid viscosity analysis (RVA) to investigate the peak viscosity of the product. This is linked to the highest level of swelling power exhibited by its starch granules.
5. Whiteness degree analysis to compare the whiteness level of the product and that of natural rice using the Colourimeter Instrument Euclidean (CIE).
6. Fourier transform-infrared (FT-IR) analysis to identify the functional groups present in the material matrix.
7. Micro-structural profile analysis using a digital optical microscope (DOM).

Among these analyses, the data obtained from the first five were subjected to ANOVA followed by multiple comparison analysis, based on Duncan's multiple range test (DMRT).

Starch Content Analysis

A sample of approximately one gram is hydrolysed with 100 ml of 3% HCl in an autoclave for 15 minutes at a temperature of 115 °C. It is then neutralised by adding NaOH 4N and diluted with distilled water to a final volume of 250 ml at pH 7, followed by filtration. A 10 ml aliquot of the filtrate is pipetted and transferred into an Erlenmeyer flask containing 25 ml of Luff Schroll solution. The mixture is heated in an autoclave or with a reflux condenser for 10 minutes until boiling. The mixture is cooled with running water, and 20 ml of 20% KI and 25 ml of 25% H_2SO_4 are added slowly. The mixture is titrated with $Na_2S_2O_3$ 0,1 N until a pale-yellow solution is formed, then 1% starch indicator is added (resulting in a blue solution). The titration continues until the blue colour disappears (denoted as a ml). A blank solution is also titrated (denoted as b ml). The starch content is calculated using Equation 1.

$$\text{Starch Content} = \frac{a \; x \; P \; x \; 0{,}95}{mg \; sample} \text{ x } 100\% \qquad [1]$$

where: P = 25 (constant numerical value)
a = volume of solution titrated

The formulation analysis aimed to evaluate the physical attributes of analogue rice (AR), focusing on its flavour, resemblance to natural rice, and lack of irritants or excessive saltiness.

Method of Production

In this study, the AR production process was carried out using an extrusion machine for the mixing, cooking, kneading, crushing, and forming or moulding processes, as recommended by (Ridwansyah et al., 2020) and (Mishra et al., 2012). AR's production process began with preparing IK flour, potato flour, and sago starch. The percentage combination is mentioned in Table 1.

These ingredients, along with GMS and water, were mixed thoroughly using a mixer for 20 minutes to ensure homogeneity. The resulting mixture was then processed in a double-screw extruder, operated at 96 °C and a speed of 168 revolutions per minute, for 30 minutes. During extrusion, a constant temperature and auger pressure were maintained. After extrusion, the AR was dehydrated in an oven at 45 °C for 30 minutes and then packaged in plastic wrapping for storage.

Determination of Physical Properties

The viscosity of the analogue rice is determined using a Brookfield viscometer model RTV. The viscosity value, expressed in centipoise, is obtained by multiplying the factor on the instrument by the measured value. 2 grams of rice are added to 10 ml of water and stirred, then 90 ml of boiling water is added and cooled until it reaches room temperature. The spindle used is spindle number 4 with a speed of 6 revolutions per minute, and the conversion factor is 1000. This is the procedure for measuring viscosity using a Brookfield RVT viscometer. (i) Attach the spindle as needed, (ii) Pour the sample to be measured into

Table 1
Four experimental formulations for analogue rice production

Formula	IK (%)	Potato (%)	Sago (%)	GSM (%)	Water (%)
I	37.00	27.00	17.00	2.00	17.00
II	27.00	22.00	12.00	2.00	37.00
III	37.00	22.00	12.00	2.00	27.00
IV	39.50	19.50	9.50	2.00	29.50

a beaker and place it under the spindle. Then lower the spindle into the sample by turning the screw at the back, (iii) Adjust the speed setting as required, (iv) Turn on the power and observe the needle; the red needle should be exactly at the 0-100 mark for the specified time (dial reading), (v) Multiply the factor by the dial reading. Factor = speed x spindle number. Example: speed = 30, spindle number = 2, so the factor is 60. To read the dial, press the small lever at the back downward. The speed setting is on the upper dial, and the spindle number is inside the box.

The whiteness degree analysis used a precise colourimetre called Chromametre CR-400 Konica Minolta (Tokyo, Japan), with the BCSF and UCSF samples in the hole through which the light passes, so that no light escapes, and then recording the values of L*, a*, and b*. Whiteness is calculated using Equation 2 (Amalia et al., 2020).

$$W = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{½.} \quad [2]$$

where, W = whiteness, L = Brightness level, between 0 (black) and 100 (white), determined based on visual observation; Δa* = Total reddish/greenish colour; Δb* = Total yellowish/bluish colour.

The micro-structure profile from the dried analogue rice sample was placed on a metal stub with double-sided adhesive tape and then coated with gold powder under vacuum to make the sample conductive. The morphology property of analogue rice was observed by using a scanning electron microscope (SEM Philips XL30), and the image was taken at an acceleration potential of 20 kV (Ashri et al., 2014).

RESULTS AND DISCUSSION

As summarised in Table 2, the amylum and amylopectin content varied across the formulas, with Formula IV showing the lowest levels. However, no significant difference was observed in the amylose content across the four experimental analogue rice formulas. The content level is mainly influenced by the amounts of IK, potato, and sago used, as their starch properties determine the final composition. Potato starch has a lower amylose content than other starches, so the percentage of potato in each formula impacts the ratio of amylose to amylopectin (Lal et al., 2024). For example, Formula I, with 27% potato, likely has a higher amylopectin content than Formula IV, which contains only 19.5% potato. Sago starch, rich in amylopectin, also increases amylopectin levels in the formula (Sumardiono et al., 2021). A higher sago content, such as 17% in Formula I, results in more amylopectin, while a lower sago content, like 9.5% in Formula IV, reduces amylopectin levels. Lower amylopectin levels improve AR quality, making Formula IV, which demonstrated the lowest amylopectin content, the optimal choice for producing high-quality and healthy AR. Based on these findings, Formula IV, which exhibited the lowest amylopectin content and optimal quality characteristics, has been selected for further analysis.

Table 2
Percentage of Amylum, Amylose, and Amylopectin in four experimental analogue rice formulas

Formula	Amylum (%)	Amylose (%)	Amylopectin (%)
I	71.63 a[z]	29.87 a	41.76 a
II	64.99 b	27.07 a	37.92 b
III	60.70 b	25.55 a	34.52 b
IV	54.92 c	24.91 a	30.01 c

Note. [z]Mean within a column followed by a different alphabet is significantly different at $p \leq 0.05$ using the LSD test

Proximate Analysis

This analysis ascertains the chemical composition of the AR produced using Formula IV by comparing IK to potato and the AR to CAR. Table 3 highlights key findings from the analysis. IK was found to have a higher crude fibre content (6.22%) compared to potato (4.55%), while potatoes exhibited significantly higher carbohydrate content (92.80%) than IK (73.58%). These characteristics suggest that producing AR using Formula IV would result in a product rich in both dietary fibre and carbohydrates, aligning with the attributes of nutritious food. Additionally, the level of calcium oxalate, a compound responsible for causing itching, was significantly reduced after immersion in a 4% NaCl solution for 48 hours. This reduction underscores the importance of addressing chemical constituents like oxalates during food processing. Factors such as cultivar, geographic location, plant maturity, and environmental conditions play critical roles in influencing oxalate levels (Chisenga et al., 2019), emphasising the need for a comprehensive understanding of these variables to optimise food safety and quality.

Table 3 presents the results of the analysis. In this table, we see that:

1. IK has a greater amount of crude fibre compared to the potato. Conversely, potatoes have a higher amount of carbohydrate content compared to IK. More specifically, the fibre content of IK is 6.22%, and that of potato is 4.55%. Meanwhile, the carbohydrate content in potato is 92.80%, much higher than the 73.58% in IK. Thus, if AR is produced using Formula IV, it will yield a substantial amount of dietary fibre and carbs. This is an exemplary characteristic of nutritious food.
2. The level of calcium oxalate, which is responsible for itching, fell dramatically when the subjects were immersed in a 4% NaCl solution for 48 hours.

Particular emphasis is placed on oxalate content. In food processing, it is crucial to comprehend the elements that influence chemical constituents like oxalates, as many factors influence oxalate content, including cultivar, geographic location, plant maturity, and environmental conditions (Chisenga et al., 2019).

Table 3
Chemical composition (in percent) of IK, potato, AR, and CAR

Object	Water	Ash	Protein	Fat	Carbohydrate	Crude Fibre	Ca-oxalate	Calcium
IK	8.84 b	8.72 a	3.05 c	0.23 d	73.58 b	6.22 a	3.21 a	1.89 a
Potato	3.71 d	2.50 c	2.20 d	1.16 c	92.80 a	4.55 c	0.80 b	0.38 c
AR	11.80 a	5.06 b	6.18 a	1.91 b	75.00 b	5.34 b	0.88 b	1.53 b
CAR	7.56 c	2.88 d	4.80 b	2.20 a	82.95 ab	4.30 c	0.65 b	1.66 b

One method to mitigate the impact of oxalates is through the cooking process (Hang et al., 2013). However, in this research instead of using boiling and fermenting methods to decrease the ca-oxalate level, the IK tubers were instead soaked in a NaCl solution of 4% concentration (or 40 gramme (g)/litre (lt)) for a duration of 48 hours. The outcome was highly significant, whereby the ca-oxalate concentration in the soaked tubers was 315 parts per million (ppm), whereas in the control (non-soaked) tubers it was 1000 ppm.

Meanwhile, while the generalised conclusion of (Hasbullah et al., 2023) was that the quality of AR produced using extruders is contingent upon the composition of the raw materials, this research goes further by coming up with a specific composition formula. The significant contribution of this research lies in the development of Formula IV, where the combined dietary fibre content of the AR produced is 35.10%, an amount far greater than the threshold value of 6% for food to be considered a source of fibre (Valencia & Purwanto, 2020).

Physical Characteristics Analysis

A crucial aspect of AR is its physical attributes, such as brightness level (whiteness scale) and the amount of sulphite residue. To measure these attributes, a physical characteristic analysis was conducted, and the findings are presented in Table 4.

As shown in Table 4, the amount of sulphite remaining in the AR was less than 3 ppm, which is the maximum allowable limit (Cuenca et al., 2008). However, in terms of the whiteness scale, the AR produced using Formula IV has a whiteness scale of 46.20%, which is numerically lower than the natural hue of white rice, which is 63.87 percent. The NaCl residue (0.93%) was also still high, perhaps due to the immersion treatment in the production process of the IK flour. Further investigation is required to reduce this residue.

Rapid Viscosity Analysis

The RVA analysis is to determine viscosity changes caused by temperature treatment. Here we summarise the viscosity profiles of the AR and CAR, together with the speed spindle. The findings show that CAR required a maximum duration of 130 seconds and a thickening

or gelatinisation temperature of 63.5 °C. However, at a thickening temperature of 69.7 °C, the peak time required for the AR was higher than that for CAR, i.e., 159 seconds. These results suggest that the heating time for starch paste in CAR is lower compared to that in the AR. The deceleration of the coagulation process in CAR led to delayed attainment of maximum viscosity.

Gelatinisation temperature refers to the specific temperature at which the viscosity of a substance increases as a result of the starch granules expanding. An increase in gelatinisation temperature signifies that the starch requires lengthier cooking duration and a higher amount of thermal energy. Consequently, the higher the gelatinisation temperature, the more stable the starch molecular crystals. This research shows that CAR requires more cooking time and thus more energy.

As mentioned in Moorthy (2002), when starch granules are heated above the gelatinisation temperature in the presence of water, they absorb a significant quantity of water and increase in size many times compared to their initial size. These findings are summarised in Table 5.

In Table 5, we see evidence of the viscosity of the AR produced using Formula IV, whether cold paste viscosity (CVP), viscosity beginning plateau (VBP) or viscosity end plateau (VEP), they were all significantly lower for the AR compared to CAR. Since VEP reflects starch's capacity to create a dense paste or gel after undergoing heating and chilling (Ashri et al., 2014), it is seen that CAR generated starch granules of greater size compared to AR, as intended. This indicates that the quality of the AR produced using Formula IV is as desired.

Table 4
Results from physical characteristic analysis

Object of Study	Sulphite (ppm)	Whiteness Scale (%)	Thickness (ºE)	NaCl Residue (%)
AR	2.30 b	46.20 a	1.57 b	0.93 a
CAR	2.80 a	38.30 b	2.11 a	0.53 b

Table 5
Results issued from RVA

Object of Study	PT: Temperature (°C) at 20cP	PT: Time (s) at 20cP	Cold Paste Viscosity (cP)	Viscosity Beginning Plateau (cP)	Viscosity End Plateau (cP)
AR	69.75 a	159.50 a	1618.00 b	135.00 b	475.50 b
CAR	63.50 a	129.50 b	4227.00 a	700.00 a	1673.00 a

Note. cP = centipoise; PT = Past Time

Further, flavour is predominantly influenced by amylose and amylopectin, two components which play a crucial role in the formation of granules. Specifically, the lower the amylose or amylopectin content, the more significant the swelling capacity and thus the better the quality of the AR. As seen in Table 2, the lower amylose and amylopectin content was from Formula IV. Moreover, as can be seen in Table 5, statistical analysis using ANOVA shows that:

1. AR required a higher temperature (69.75 °C) than CAR (63.50 °C) (shown in the first column).
2. AR required a longer duration (159.50 seconds) than CAR (129.50 seconds) (refer to column 2).
3. The cold paste viscosity (CPV), also called the coefficient of apparent viscosity, of the AR was significantly lower (1618.00 cP) than that of CAR (4227.00 cP). The VBP of the AR was also lower (135.00 cP) than that of CAR (700.00 cP).

Whiteness Degree Analysis

The results are presented in Table 6. Further analysis using ANOVA reveals that at 5% significance level, there is a significant difference between the whiteness level of the AR and that of PR. This is another important finding of this research. The disparity in percentages (40.10% for PR and 32.07% for AR) can be attributed to the light brown colour of the IK flour. In fact, the whiteness index of the AR is entirely dependent upon the whiteness index of the IK chips. The heating during the extrusion process can cause gelatinisation of the carbohydrate ingredients, which may reduce the whiteness level of the analogue rice product (Amalia et al., 2020).

Table 6
Whiteness degree (%) of PR and AR

Component	Whiteness
PR	40.10 a
AR	32.07 b

FT-IR Analysis

In this research, the functional groups of the AR produced using Formula IV were examined using FT-IR analysis. Then, as a comparison, that of IK, potato, CAR and PR were also examined. It is expected that functional groups with similar characteristics will exhibit the same chemical reactions. The graphical results are displayed in Figure 1, while the numerical results are shown in Table 7.

Figure 1 shows that, in terms of absorbance (in %) with respect to wave number (in cm^{-1}), the pattern for IK is the same as that for potato, AR, CAR, and PR. The numerical data in Table 7 adds weight to the patterns shown in Figure 1.

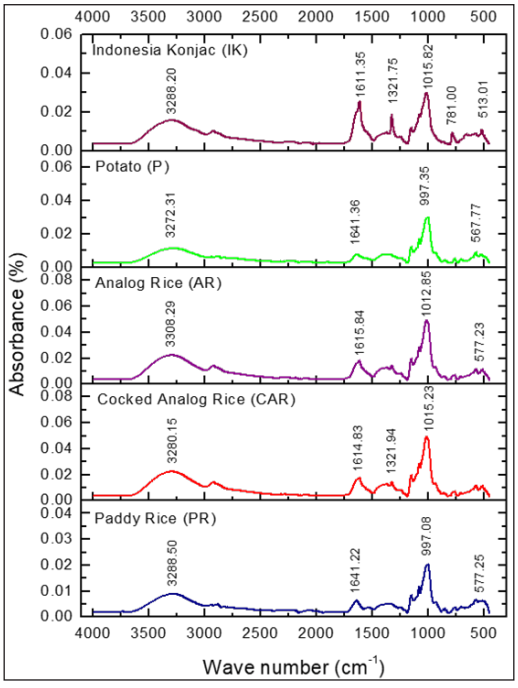


Figure 1. FT-IR spectra of IK, potato, AR, CAR, and PR (top to bottom)

Microstructure Profile Analysis

In order to analyse the microstructure of both the AR and CAR, the scanning electron microscope (SEM Philips XL30) and the image was taken at an acceleration potential of 20 kV was employed. Examination was at a magnification, which enabled accurate determination of the presence of porosity and starch granules. The results are displayed in Figure 2: (a) microstructure profile for AR, and (b) for that of CAR. This figure illustrates the contrasting visual characteristics of AR and CAR with regard to porosity and gelatinisation (Ashri et al., 2014).

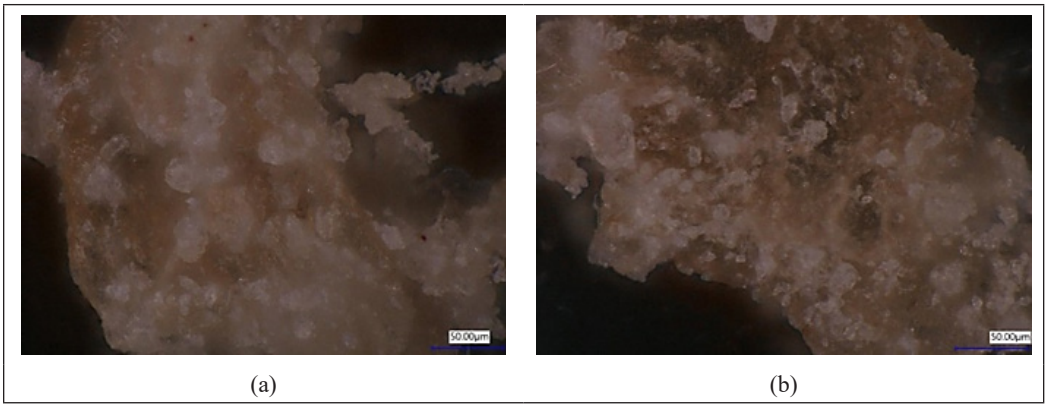
The results suggest that the gelatinisation of the AR and CAR led to starch degradation and retrogradation. Additionally, unlike the AR, the character of CAR shifted to a high porosity level, which means that it became absorbent. This strengthens the findings of (Mendoza et al., 2012).

CONCLUSION

In the attempt to produce high-quality AR, this research came up with Formula IV, which gives the optimal composition of IK flour, potato flour, sago, GMS, and water. The formula IV on the best composition were: Indonesia konjac (39,50 %), *S. tuberosum* or potato (19,50 %), sago (9,50 %), glyceryl monostearate (GSM) (2,00 %), and water (29,50 %).

Table 7
Numerical results in terms of wavelength (cm^{-1}) and vibration type

PR		CAR		AR	
Wavelength	**Vibration Type**	**Wavelength**	**Vibration Type**	**Wavelength**	**Vibration Type**
577.25	CH bend bonded	-	-	577.24	CH bend bonded
997.08	C-O-C stretch	1015.23	C-O-C stretch	1012.85	C-O-C stretch
1641.22	C=C stretch, C=O stretch	1614.83	C=C stretch, C=O stretch	1615.8	C=C stretch, C=O stretch
3288.50	OH stretch, H-bonded	3280.15	OH stretch, H-bonded	3308.29	OH stretch, H-bonded



(a) (b)

Figure 2. Microstructure profile of (a) AR and that of (b) CAR

The AR produced using this formula (IV) has been achieved and can be categorised as a dietary food, which is an important discovery.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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The Influence of Zinc and Boron Application on Growth, Yield, Seed Quality, and Nutrient Content in Non-hybrid Corn

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ABSTRACT

Zinc (Zn) and Boron (B) are micronutrients required by plants in small amounts but play an important role in plant growth. Zn and B contents are also essential nutrients for human health. There has been no research on the effects of Zn and B as biofortification on the Srikandi variety corn (non-hybrid). This study aimed to determine the effect of Zinc and Boron spraying on growth, productivity, seed quality, and Zn and B content in the Srikandi variety. This experiment consisted of four treatments of Zinc and Boron spraying on leaves, namely (1) Zinc 0% + Boron 0%; (2) Zinc 0.5% + Boron 0.5%; (3) Zinc 1% + Boron 1%; (4) Zinc 2% + Boron 1.5%. The results showed that Zn and B applications significantly affected the growth, seed quality, and nutritional content of the Srikandi variety corn. Application of Zn 1% + B 1% was the best treatment for increasing the number of leaves, stover weight, seed germination and vigour, growth homogeneity, and Zn and B content in corn.

Keywords: Biofortification, boron, corn, nutrition, seed, zinc

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INTRODUCTION

Corn is one of the important food crops in Indonesia. Corn consumption as food in Indonesia continues to increase from 2021-2023, respectively, 2.7 kg cap^{-1} $year^{-1}$; 3.1 kg cap^{-1} $year^{-1}$; 3.6 kg cap^{-1} $year^{-1}$. Increased corn consumption must be accompanied by increased production to meet corn needs. According to Edy (2020), the Srikandi corn variety was one of the

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superior varieties that was quite popular in Indonesia because of its high productivity and superior nutritional value. This variety is included in the Quality Protein Maize (QPM) corn group, which has a higher content of the essential amino acids lysine and tryptophan than regular corn, making it useful for improving the nutritional quality of food and feed.

Zinc (Zn) and boron (B) are essential micronutrients that play an important role in corn plant growth and human health. Deficiencies of these two elements in plants reduce corn's nutritional quality, especially in the context of preventing stunting in children (Praharaj et al., 2021). Deficiencies of Zn and B cause stunted plant growth, decreased productivity, low grain quality, and low Zn, B, and protein content (Singh et al., 2020). Zn and B are micronutrients that play an important role in plant growth and production. Zn is important in various enzymatic reactions, nitrogen metabolism processes, energy transfer, and protein synthesis (Choukri et al., 2022; Kumar et al., 2019; Singh et al., 2020). Zn deficiency in plants is seen in the form of symptoms of stunted growth, chlorosis, shrunken leaves, and empty grains (Suganya et al., 2020). In corn plants, Zn application can increase growth and yield (Basavanneppa et al., 2020; Hisham et al., 2021; Mohsin et al., 2014; Palai et al., 2018; Stepić et al., 2022).

Boron is one of the nutrients that also plays an important role in plant growth and reproduction. B played a role in carbohydrate metabolism and sugar transport, plant cell wall formation, lignification, RNA metabolism, indole acetic acid (IAA) metabolism and pollen tube growth (Nogueira et al., 2019; Santra et al., 2024). B plays an important role in increasing corn yields, as it significantly affects the number of seeds per cob, cob length, and harvest yield (Almosawy et al., 2019). B applied with plant growth regulators increased corn yields (Gurjar et al., 2020). Bayar et al. (2024) reported that spraying corn plants with 1% B increased plant height, leaf area, yield, and 1000-grain weight. B deficiency in plants caused the cessation of terminal shoot growth, death of young leaves, inhibition of sugar transport, and formation and germination of pollen. Seeds and grains were also reduced if the boron supply was hampered (Jolli et al., 2020).

In humans, Zn is needed to boost the immune system. Zn played an important role in enzyme activation and body metabolism (Suganya et al., 2020). Zn deficiency in children disrupts physical growth or stunting, the immune system, learning ability, and the development of cancer cells (Abdoli & Esfandiari, 2017; Boonchuay et al., 2013; Shori et al., 2019). B is a mineral that plays an important role in several human biological processes. B showed various pleiotropic effects, ranging from anti-inflammatory and antioxidant effects to the immune system, affecting bone growth and central nervous system function, relieving rheumatic symptoms, affecting hormone function, and reducing the risk of several types of cancer (Meacham et al., 2010; Nielsen, 2014).

Zn and B in plants plays role in photosynthesis, protein synthesis, chlorophyll, carbohydrates, flowering regulation, and reducing kernel abortion (Kumar et al., 2019).

Singh et al. (2020) revealed that applying Zn and B increased plant height, stem circumference, cob length, and corn grain index. The combined application of Zn and B resulted in higher growth because both elements play important roles in the process of assimilation rate and metabolic activity in plants (Sharma & Mehera, 2022). Mohsin et al. (2014) suggested that the combined application of Zn, such as seed priming (2.0%) and foliar spraying (2.0%), can improve the performance of hybrid corn. Foliar application in corn at concentrations of approximately 0.5–1.5% $ZnSO_4$ and H_3BO_3 has been shown to improve nutrient status and seed quality without causing toxicity (Mohsin et al., 2014; Suganya et al., 2020; Singh et al., 2020). According to Fageria et al. (2009), foliar fertilisation is often more economical, rapidly absorbed, and effective in improving plant nutrient status compared to several other approaches. Based on the literature study, we want to know the highest Zn and B content from the application of a combination of Zn and B on the Srikandi variety corn that we have compiled.

MATERIALS AND METHODS

The research was conducted from May-September 2023. Field trials were conducted at the Lampung State Polytechnic Experimental Garden. Seed quality testing was conducted at the Seed and Plant Breeding Laboratory, Faculty of Agriculture, Lampung University, Indonesia. Zn and B content analysis was conducted at the Integrated Laboratory and Technology Innovation Centre, Lampung University. The experiment was conducted in a Randomised Block Design (RBD) consisting of 4 treatments with 3 replications. The treatments were a combination of Zn and B, namely (1) Zn 0% + B 0% (control); (2) Zn 0.5% + B 0.5%; (3) Zn 1% + B 1%; (4) Zn 1.5% + B 1.5%.

Plant Preparation

Local corn variety Srikandi Ungu is planted with a spacing of 0.7 m x 0.2 m. Seed planting was done by digging holes with 2 corn seeds per planting hole. Fertilisation was given with the respective doses: 150 kg/ha urea, 100 kg/ha SP36 (Phosphate 36%), 100 kg/ha KCl. KCl and Phosphate fertilisers were given entirely one week after planting. Urea fertiliser was given 3 times at the 7th, 21st, and 45th days after planting. The soil used had a pH of 8.32 with a Nitrogen content of 0.10%, P = 193.55 ppm, K = 11.55 ppm, Zn = 53.27 ppm, and B = 77.76 ppm.

Application of Zn and B

The chemicals $ZnSO_4.7H_2O$ and H_3BO_3 were used as sources of Zn, and B. Zn and B were applied by spraying twice, namely at 30 and 50 days after planting (Singh et al., 2020).

Harvesting was carried out when the corn plants were 87 days after planting or when the corn had reached physiological maturity.

Observation Variable

The variables observed in this study were plant growth, harvest yield, seed quality, and zinc and boron content. Plant height was measured from the root neck to the tip of the highest leaf. The number of leaves counted was the number of corn leaves that had opened perfectly. Chlorophyll content in leaves was measured using a Chlorophyll Content Meter (CCM) brand Opti Sciences type CCM-200. The weight of cobs with and without husks, the number of rows per cob, the number of seeds per row, and the number of seeds in one cob were measured at harvest time, 87 days after planting. Physiological quality was measured according to the 2021 International Seed Testing Association guidelines. The Zn and B content in corn seeds was analysed using the Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) instrument. Measurements were carried out on ten sample plants per experimental plot.

Data Analysis

The data obtained were analysed for variance, followed by the Least Significant Difference test at α 5%. Data analysis was carried out using the R Studio data processing programme.

RESULTS AND DISCUSSION

Effect of Zn and B Spraying on Plant Growth

The application of Zn and B increased the number of leaves and stover the weight. The application of Zn 1% + B 1% was the best treatment in increasing the growth of Srikandi variety corn plants compared to other treatments on the variables of the number of leaves and the wet and dry stover weight in Table 1.

Table 1
Effect of Zn and B spraying on the growth of Srikandi variety corn

Treatment	PH (cm)	NL	TLA (cm^2)	CCI	CC ($\mu M\ m^{-2}$)	WSW (g)	DSW (g)
Control	237.2[a]	14.10[c]	438.10[a]	32.80[a]	436,3[a]	379.3[d]	132.5[c]
Zn 0.5% + B 0.5%	248.5[a]	14.75[b]	489.06[a]	36.51[a]	463,4[a]	592.7[bc]	219.8[ab]
Zn 1% + B 1%,	260.4[a]	15.17[a]	500.98[a]	34.06[a]	446,6[a]	615.5[a]	257.8[a]
Zn 1.5% + B 1.5%	239.2[a]	14.58[b]	530.51[a]	32.89[a]	437,6[a]	503.3[c]	188.7[bc]

Note. [1]Numbers followed by the same letter do not differ based on the LSD test at 5% levels. PH = Plant Height; NL = Number of Leaves; TLA = Total Leaf Area; CCI = Chlorophyll Content Index; CC = Chlorophyll Content; WSW = Wet Stover Weight; DSW = Dry Stover Weight

The increase in plant growth caused by applying Zn and B is evident from increased in chlorophyll content, the maximum number of leaves, and accumulation of dry matter in plants. This is supported by Tavallali (2017), who stated that the application of Zn and B had a synergistic effect in increasing plant dry weight, photosynthesis, and chlorophyll content. The application of Zn and B simultaneously provides higher growth results because both elements play an important role in the process of assimilation rate and metabolic activity in plants (Sharma & Mehera, 2022). The increase of Zn and B content was due to the influence of metabolic activity and enzymes that increase photosynthesis, leading to increased vegatative plant growth (Faiyad et al., 2023; Kumar et al., 2019). In this study, the insignificance of chlorophyll may have been caused by the accumulation of chlorophyll having been used optimally in increasing the number of leaves and stover weight of corn during the observation period.

Effect of Zn and B Spraying on Yield

The application of Zn and B to the Srikandi corn variety did not differ significantly from the control in terms of the productivity variables of corn cobs and kernels (Table 2). However, Srikandi variety corn is a superior type of Indonesian corn that has high productivity far above the average national corn productivity. This condition indicates that the increase in source capacity (photosynthesis and vegetative biomass) was not offset by an increase in sink strength during the generative phase, such as grain number and filling capacity. In the genetically high-yielding Srikandi variety, the sink potential may have approached its physiological limit, resulting in additional assimilates being allocated more to vegetative tissues, reflected in the relatively unchanged harvest index. This phenomenon is consistent with reports that micronutrient responses are often stronger in the vegetative phase and plant physiological quality compared to quantitative yield increases, especially when there is no severe nutrient deficiency (Singh et al., 2020; Sharma & Mehera, 2022). Based on the adaptation test by Wahid et al. (2022), Srikandi corn had high adaptation to dry land

Table 2
Effect of Zn and B spraying on the production of Srikandi variety corn

Treatment	Cob Fresh Matter with Husk (g)	Cob Fresh Matter without Husk (g)	Row per Cob (rows)	Seed per Row (seeds)	Seed per Cob (seeds)	1000 Seed Weight (g)
Zn 0% + B 0%	246.2[a]	204.1[a]	13.33[a]	27.0[a]	359.33[a]	258.70[a]
Zn 0.5% + B 0.5%	289.3[a]	237.4[a]	15.22[a]	29.0[a]	444.78[a]	271.37[a]
Zn 1% + B 1%,	290.3[a]	249.2[a]	16.11[a]	32.9[a]	531.37[a]	264.29[a]
Zn 1.5% + B1.5%	230.3[a]	189.1[a]	14.11[a]	27.1[a]	380.85[a]	242.91[a]

Note. [2]Numbers followed by the same letter do not differ based on the LSD test at the 5% level

with higher productivity compared to other new superior corn varieties, namely Gumarang, Lamuru, and Bisma. Although the application of Zn and B to Srikandi corn plants did not affect corn yields, this variety already had higher yields than other corn varieties. In addition, the application of Zn and B to the Srikandi variety corn affected other parameters, such as growth (see Table 1), seed quality (see Table 3), and corn nutrition (see Table 4). The results align with the research of Singh et al. (2020), which explains that applying 1% Zinc and 1% Boron increases the diameter of the cob, seeds per row, rows per cob, and seed index.

Zinc is a micronutrient needed in metabolic activities that play a role in ensuring the quality of plant productivity (Tondey et al., 2022). Zinc played a role in carbohydrate metabolism, protein synthesis, and the regulation of auxin synthesis (Kumar et al., 2019). Boron helps the development of fruits and seeds, sugar transport, and hormone development (Kumar et al., 2019). John et al. (2011) revealed that the application of Boron to plants has an important effect on transporting sugar in photosynthesis, increasing flower production, fruit ripening, seed retention and development. Kumar et al. (2019) reported that spraying sweet corn with 0.3% B increased plant height, dry weight, and plant height growth rate. In the production attribute, boron increased the number of cobs per plant, the number of seeds per cob, the number of rows per cob, the seed index, and production.

Effect of Zn and B Spraying on Seed Quality

Seed quality was improved by the application of Zn 1% + B 1%. The application of Zn 1% + B 1% and Zn 1.5% + B 1.5% increased the percentages of seed vigour and growth homogeneity of the Srikandi variety corn. In addition, seed germination was also improved by application of Zn 1% + B1% (Table 3). Germination was an important phase to ensure optimal plant production and productivity (Tondey et al., 2022). In addition, germination was also an important stage in determining whether the seeds planted would have strong vigour and viability (Sedghi et al., 2013). The application of Zinc and Boron interacts with each other in the germination process.

Table 3
Effect of Zn and B spraying on the quality of Srikandi variety corn seeds

Treatment	Germination (%)	Vigour Index (%)	Growth Homogeneity (%)	Growth Speed (% day-1)	Maximal Potential Growth (%)
Zn 0% + B 0%	80[b]	78[b]	80[b]	17.3[a]	96[a]
Zn 0.5% + B 0.5%	86[b]	84[b]	86[b]	17.1[a]	98[a]
Zn 1% + B 1%,	98[a]	98[a]	98[a]	19.6[a]	100[a]
Zn 1.5% + B1.5%	82[b]	82[a]	82[a]	16.4[a]	98[a]

Note. [3]*Numbers followed by the same letter do not differ based on the LSD test at the 5% level*

The concentration of Zinc given to plants affects germination (Basha & Selvaraju, 2015). The application of Zn 1% + B 1% had the highest vigour index, germination power, and growth homogeneity of 98%. Lemes et al. (2015) reported that providing Zn increased shoot length by 9.3% and root length by 5.1%.

According to Ahmad et al. (2009), Zn has functions related to the strength and development of cell walls, seed development, hormone development, and is important in plant growth, such as in the development of leaves and leaf buds. Meanwhile, Zn increased metabolic activity and helped the growth of plant embryos during germination (Deepika, 2015). Zinc also played a role in protein synthesis, which played a role in good seed development, germination power, and high seed vigour index (Geetharani et al., 2008).

Abdoli & Esfandiari (2017) revealed that high Zn content in seeds can increase the vigour index and germination rate compared to seeds with low Zn content. The results showed a relationship between Zn content and germination; seeds containing high Zn significantly increased the percentage of root germination by 10.1% and the seedling vigour index by 26%. Zn application through leaves had been shown to increase Zn content in seeds more than Zn application in soil (Suganya et al., 2020).

Effect of Zn and B Spraying on Zn and B Corn Nutrient Content

The application of Zn and B increased the Zn content in the Srikandi variety corn. However, only the concentration of 1% Zn + 1% B also increased the B content in corn; other Zn and B treatments even reduced the B content (Table 4). According to Pawlowski et al. (2019) reported that corn with high Zn levels caused B levels in leaves and seeds to decrease, while in low Zn treatment, the opposite effect occurred. Excessive doses of Zn application also caused a decrease in B levels in corn leaves in greenhouses, although field experiments did not show any relationship between Zn application and B levels (Aref, 2012).

Table 4
Effect of Zn and B spraying on Zn and B content of Srikandi variety corn

Treatment	Zn Content in Corn (mg kg-1)	Boron Content in Corn (mg kg-1)
Zn 0% + B 0%	22.80[c]	0,49[b]
Zn 0.5% + B 0.5%	58.10[a]	0,07[d]
Zn 1% + B 1%,	35.48[b]	0,99[a]
Zn 1.5% + B1.5%	41.20[b]	0.39[c]

Note. [4]*Numbers followed by the same letter do not differ based on the LSD test at the 5% level*

CONCLUSION

Application of Zn 1% + B 1% on Srikandi variety corn plants was the best treatment in increasing growth through increasing the number of leaves and stover weight. Although the application of Zn + B did not increase yields, the application of Zn 1% + B 1% improved seed quality by increasing germination, seed vigour and growth homogeneity. In addition, the application of Zn 1% + B 1% also increased the Zn and B content of corn.

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A Second Life for Agro-industrial Residues: Edible Coating from Roselle Decoction Residues for Preserving Wax Apple Quality and Antioxidant Properties

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ABSTRACT

Postharvest deterioration and fungal infection significantly limit the shelf life of wax apples (*Syzygium samarangense*), while roselle decoction residues remain underutilised despite retaining bioactive compounds after processing. This study aimed to evaluate the efficacy of roselle decoction residues as functional ingredients in edible coatings for wax apple preservation. Fresh roselle calyx (RC) (F3), fresh decocted cordial residue (DCR) (F4), freeze-dried RC (F5), and freeze-dried DCR (F6) were incorporated into various coating formulations and applied to fruits stored for 15 days at ambient temperature. Roselle-based coatings significantly delayed fruit deterioration by preserving colour, firmness, and biochemical attributes (soluble solids, acidity, vitamin C, phenolics, and flavonoids) compared to controls. Water loss was reduced, and antioxidant capacity (DPPH) was better maintained in coated fruits. Among treatments, fresh RC (F3) exhibited the most consistent performance in preserving quality attributes, followed by F6, F4, and F5. Under wound-inoculation conditions, selected roselle-based coatings also delayed visible disease development caused by *Pestalotiopsis* sp. Overall, the findings demonstrate that roselle decoction residues can be valorised as natural antioxidant-rich coating agents, contributing to improved postharvest quality and supporting sustainable by-product utilisation.

Keywords: Antifungal effect, antioxidant activity, cordial by-products, edible coating, fruit preservation, postharvest quality, roselle residue, waste valorisation

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INTRODUCTION

The postharvest handling process faces several obstacles, and in developing regions, these are even more pronounced due to limited storage capabilities and inadequate handling procedures, which continue to result in loss of the food supply chain. In the Asia-Pacific region, postharvest losses are primarily the result of physiological breakdown, microbial infestations, and mechanical disruptions throughout the distribution and marketing processes. Moist, fresh, and highly perishable fruit is especially susceptible to deterioration and is quickly degraded under the conditions found at local markets in developing regions (Esua et al., 2017; Food and Agriculture Organisation [FAO], 2011; Roelle, 2014).

Of the numerous postharvest handling challenges, fungal infections are perhaps the most destructive and have the greatest economic impact on the producer. In the postharvest handling process, several environmental conditions, such as high temperature and low humidity, in conjunction with physical handling stress, significantly promote fruit deterioration and ultimately result in the complete loss of marketable shelf life (Mendy et al., 2019; Shama et al., 2024). When weighing the costs associated with postharvest interventions, especially for small-scale fruit producers, managing marketable shelf life remains critical. Edible coatings (Vilaplana et al., 2020) provide a viable, low-cost, and potentially effective means of helping to reduce shelf-life-dependent postharvest losses.

Wax apple (*Syzygium samarangense*) is a highly perishable tropical fruit. Mechanical damage, high vapour pressure deficit, and high moisture content lead to rapid moisture loss and microbial decay. In Malaysia and other tropical countries, wax apples are sold at ambient temperatures, which is conducive to rapid fungal infections and shortening of shelf life (Department of Agriculture [DOA], 2022; Fenta et al., 2023; Sridhar et al., 2021). This indicates the need for simple and effective preservation methods.

Edible coatings have gained traction for their ability to enhance the postharvest shelf life of fresh fruits. Coatings enhance the shelf life of fruits by decreasing the rate of water loss, regulating gaseous exchange, and slowing the rate of biochemical changes. Edible coatings create a semipermeable barrier to protect fruits and also provide a means to reduce the incidence of oxidative stress and microbial infection by incorporating natural antioxidants and antimicrobials into the coating. (Dhall, 2013; Karthi et al., 2023; Pashova, 2023; Romanazzi et al., 2017; Sharma et al., 2019). Due to their low cost and simple application, coatings are well-suited for tropical fruits.

Recently, the focus has shifted to the use of plant-based residues as functional components of coatings. Coatings that incorporate by-products of plant origin with phenolic compounds contribute to extending the postharvest life of fruits, waste valorisation, and sustainable food systems. Several researchers have demonstrated coatings with plant-based antimicrobial and antioxidant compounds, thereby reducing the need for chemical additives (Flores-Contreras et al., 2024). However, as of now, the use of plant-based materials for coatings has yet to be thoroughly researched.

Roselle (*Hibiscus sabdariffa* L.) is a commercial beverage ingredient that produces high amounts of decoction (a method of boiling) waste that is usually thrown away. Studies have shown that even after heating, waste can still have high amounts of bioactive compounds like phenols, flavonoids, and some organic acids. The extracts from the wastes of roselle have shown *in vitro* antioxidant and antifungal properties (Yusoff et al., 2024), showing that they have potential use in postharvest preservation. However, there is little knowledge of how roselle decoction waste as part of an edible coating on fresh fruit, impacts the fruit's retaining quality and inhibits disease during storage.

This is the background on which the present study is based on, to explore the use of edible coatings made from waste of roselle decoctions and to quantify, for the first time, control of quality attributes (physiochemical), antioxidant, and disease of the wax apples during storage at ambient temperature. The study also attempts to capture the potential of using waste of roselle, with the understanding that incorporating fresh and freeze-dried roselle waste would be more practical, to create non-futuristic value-added coatings.

MATERIALS AND METHODS

Plant Material

The Terengganu variety roselle calyxes (UMKL-1) were purchased from Aslah Hibiscus, Aslah Supply, and Services located in Batu Pahat, Johor, Malaysia (4.2105° N, 101.9758° E). The wax apples were obtained from Ladang Konsep Loceng Merah, Felda Lubuk Merbau, Kuala Nerang, Kedah (06°16' N, 100°37' E). Both samples were brought to the Postharvest Technology Laboratory, Faculty of Food Science and Agrotechnology, Universiti Malaysia Terengganu (5.3117° N, 103.1324° E). The selection of fruits was done in conformity with the commercial maturity index 3 and size L standards, as per the Malaysian Standard (2015) for the grading of wax apples. The outlined standards reflect and ensure uniformity of the experimental units to the uniform physiological maturity and commercial harvest stage.

Sample Preparation

The edible coatings were made using roselle calyces, which were extracted via the decoction method prior to extraction. Extraction was done via cold maceration, and then ethanol was removed using a rotary evaporator (Buchi R-300EL). Ethanol was chosen as the extraction solvent because it is considered a better solvent for the recovery of phenolic compounds, flavonoids, and other bioactive constituents of plant sources. After the rotary evaporator, the ethanol extraction was removed under pressure until it was dry prior to the extract being added to the aqueous coating formulations. Hence, the coatings were water-based and did not have any extraction solvent.

To minimise variability due to the presence of natural phytochemicals, all the roselle materials were sourced from a single supply and were all subjected to the same extraction parameters, such as solvent ratio, extraction time, and concentration. These same controlled parameters were consistently applied to preserve the relative phenolics and anthocyanins compositions prior to the formation of the edible coatings.

The experimental treatments consisted of uncoated wax apples (F1), a chitosan-based coating (F2), and roselle-based coatings formulated with 3% (w/v) fresh RC (F3), 3% (w/v) fresh DCR (F4), 3% (w/v) freeze-dried RC (F5), and 3% (w/v) freeze-dried DCR (F6). Chitosan was used as a positive control due to its extensive research as an edible coating material in postharvest studies, particularly for its film-forming, antimicrobial, and gas barrier properties, which serve as a recognised scientific benchmark for assessing innovative bioactive coatings. The 3% (w/v) concentration for all roselle-based formulations was determined from previous *in vitro* screening as the most effective in promoting stability of the formulation and inhibiting fungal growth. Higher concentrations did not induce a proportional increase in antifungal activity and negatively impacted the viscosity and uniformity of the coating, whereas lower concentrations caused a decrease in antifungal activity.

The chitosan coating (F2) was prepared using 1% (w/v) chitosan dissolved in 1% (v/v) acetic acid, followed by the addition of glycerol (20%, w/w) as a plasticizer and Tween 80 (1%, w/w) as an emulsifying agent, according to No et al. (2006) and Han et al. (2014). Roselle-based coatings (F3–F6) were prepared in aqueous solutions containing glycerol (20%, w/w) and Tween 80 (1%, w/w), without the addition of acetic acid, to avoid additional acidification that could confound the contribution of roselle-derived organic acids and bioactive compounds. Glycerol and Tween 80 concentrations were kept constant across all roselle-based formulations to standardize coating plasticity and dispersion properties.

The wax apples were washed under running tap water and air-dried on the paper towel for 2 h. The fruits were then surface-sterilized in 70 % ethanol, rinsed with sterile distilled water, and air-dried in a laminar flow at ambient temperature for 30 min. After that, the fruits were dipped in their corresponding coating solution for 30 seconds and allowed to air dry, where the coating solution was allowed to drain off. The purpose was to establish uniform coating applications across all treatments and replicates; therefore, coating application times, solution concentrations, and drain times were all standardised in controlled laboratory conditions. Coated fruits were then placed on a mesh rack and dried at room temperature. Once the coated fruits were dry, they were wounded on the equatorial surface using a sterile needle for fungal inoculation. Mycelial agar plugs obtained from 7-day old cultures of *Pestalotiopsis* sp. were carefully placed onto the wounding sites. The inoculated fruits were then kept at 25 ± 2 ºC for 15 days for analysis. The 25 ± 2 °C temperature was set to mimic first-tier customer-retail conditions, interleaving the projection

of how wax apples would be displayed and sold at room temperature (not maintained in a refrigerated condition) within the local distribution chains. This approach allows evaluation of coating performance against real-world commercial handling conditions.

Colour

Wax apple was measured by Konica Minolta Chroma-Meter using CIE L*, a* and b* system. Colour was measured at three equatorials opposite sides for each wax apple surface and expressed in lightness (L*), a* value, b* value, hue angle (h°), and chroma (C*) (Nur Amirah, 2015).

Weight Loss

Weight loss was determined by measuring the reduction in fruit weight during storage and expressed as a percentage. The initial weight of each wax apple was recorded before being stored. The final weight was measured at 0, 3, 6, 9, 12, and 15 days of storage using a weighing balance (Model B303-5, Mettler Toledo, Japan).

Firmness

Three puncture tests were made per fruit surrounding the inoculated fungi by using a puncture probe set of P2 over a metal plate. The firmness was determined by using an electronic pressure tester (Model TA-XTPlus Texture Analyser), with pre-test speed: 1.0 mm/sec, test speed: 0.05 mm/sec, and post-test speed: 1.0 mm/sec, and distance of penetration: 0.7 mm. The data were expressed in Newton (N).

Soluble Solid Concentration (SSC)

One gram of the sample was homogenised using a handheld juice extractor (blender), and the juice was filtered through muslin cloth to remove suspended solids. A few drops of the extracted sample were then applied to the refractometer prism. To ensure more accurate readings, the data were recorded three times for each experimental unit using an infrared digital refractometer (Atago-Palette PR 101, Atago Co. Ltd., Itabashi-Ku, Tokyo, Japan) (Association of Official Analytical Chemists [AOAC], n.d.).

Titratable Acidity (TA)

The samples (2 g) were homogenised with 40 mL of distilled water using a handheld juice extractor (blender). Aliquots of 5 mL of the extracted juice were filtered and titrated with 0.1 N sodium hydroxide (NaOH), using phenolphthalein as an indicator. A pH meter was used to monitor changes in the samples' pH. The titration was stopped when the pH meter indicated a value of 8.1. The volume of titrant used and any colour changes were recorded

and expressed as a percentage of malic acid. The TA was calculated as a percentage of malic acid according to Ranggana (1986).

SSC/TA ratio

The SSC/TA ratio was calculated by dividing the SSC by the TA values, with the results expressed as a percentage (%).

pH Value

The sample (2 g) was homogenised with 40 mL of distilled water using a juice extractor (handheld blender). The aliquots from extracted juice were determined by a glass-electrode pH meter using buffers of pH 4.0 and 7.0 for calibration (Nur Amirah, 2015).

Vitamin C

Vitamin C was determined according to the method of Nur Amirah (2015) and Esua et al. (2017). The 2 g of wax apple were extracted with 10 mL of metaphosphoric-acetic acid solution. The homogenate was then centrifuged at 3000 rpm for 15 minutes. A 500 µL aliquot of the supernatant was mixed with 200 µL of 3% metaphosphoric acid, 1400 mL of distilled water, and 200 µL of diluted Folin reagent (Folin distilled water, 1:5 v/v). The absorbance of the supernatant was measured at 760 nm using a UV-VIS Spectrophotometer (UV-1800, Shimadzu). The vitamin C content was expressed as mg AAE/100 g fresh weight (FW) based on a calibration curve of authentic L-ascorbic acid (0 µg/mL to 140 µg/mL; y=0.0066x+0.0509; $r2$= 0.998)

Total Phenolic Content (TPC)

The TPC was determined by the Folin-Ciocalteau method, adapted from Swain and Hillis (1959) and Thaipong et al. (2006). A total of 150 µL of sample extract, 2400 µL of pure water, and 150 µL of 0.25 N Folin-Ciocalteau reagent were combined in a cuvette and mixed thoroughly using a vortex. The mixture was allowed to react for 3 minutes. Then, 300 µL of a 1 N sodium carbonate (Na_2CO_3) solution was added to each cuvette and mixed well. The solution was incubated in the dark for 2 h at room temperature (25 °C). The absorbance was measured at 725 nm by UV-Vis spectrophotometer (UV-1800, Shimadzu) and expressed as gallic acid equivalents (GAE), mg GAE/g extract, using gallic acid (0 µg/mL to 40 µg/mL; y=0.0273x+0.0816; $r2$= 0.9904) as the standard.

Total Flavonoid Content (TFC)

The TFC was determined using a modified version of the method described by Dewanto et al. (2002) and Abu Bakar et al. (2009). A 500 µL sample extract was mixed with 2250 µL of

distilled water in test tubes. After 6 min, 150 µL of a 5% sodium nitrite ($NaNO_2$) solution was added, followed by 300 µL of a 10% aluminum chloride ($AlCl_3 \cdot 6H_2O$) solution, which was allowed to react for 5 min. Then, 1000 µL of 1 M NaOH was added, and the mixture was thoroughly vortexed. The absorbance was measured at 510 nm using a UV-Vis spectrophotometer (UV-1800, Shimadzu), and the results were expressed as quercetin equivalents (QE), mg QE/g extract. A quercetin standard solution ranging from 0 µg/mL to 125 µg/mL (y=0.0009x+0.0682; r2= 0.9996) was employed for calibration purposes.

DPPH Radical Scavenging Assay

The radical-scavenging ability of wax apple extract was evaluated to determine antioxidant activity as described by Esua et al. (2017). Initially, 500 µL of the lipophilic extract supernatant was combined with 500 µL of 0.1 mM DPPH reagent, vortexed, and placed in the dark to incubate for 30 min at room temperature. Using a UV-Vis spectrophotometer (UV-1800, Shimadzu) with methanol as a blank, the absorbance of the sample was measured at 517 nm.

Experimental Design and Statistical Analysis

The experimental design was a complete randomised design (CRD) factorial with six coating formulations (F1 to F6) and six storage durations (0, 3, 6, 9, 12, and 15 days). Each treatment combination had five replicates; the experimental unit was one individual fruit. Two-way analysis of variance (ANOVA) was used to evaluate the effects of fruit coating, duration of storage, and their interaction. Mean separation was done using Tukey's Honestly Significant Difference (HSD) test at $p \leq 0.05$. All data analysis was carried out in SAS Studio 3.81 (SAS, 2024).

RESULTS AND DISCUSSION

The interaction effects between coating formulation and storage duration (CF × SD) for colour, weight loss, and firmness suggest that storage duration effects change based on what formulation is applied. Edible coatings applied during storage have a significant effect on the colour attributes of wax apples. Key chromatic parameters (L*, a*, b*, h°, and C*) changed the most, indicating a shift in postharvest brightness, saturation, and hue (Figure 1) parameters. During storage, a redder hue in wax apples was attributed to a reduction in h° and L, and a decline in b, accompanied by an increase in a. Coated formulations performed differently in colour retention. Among coated formulations, F3 and F6 were the most colour characteristic preservers, having maintained a redder (a), better hue (h°) stability, and colour saturation (C*). For F3, this improvement in stability was accompanied by a brightness and visual vibrancy improvement. For F6, improvement in stability was accompanied by

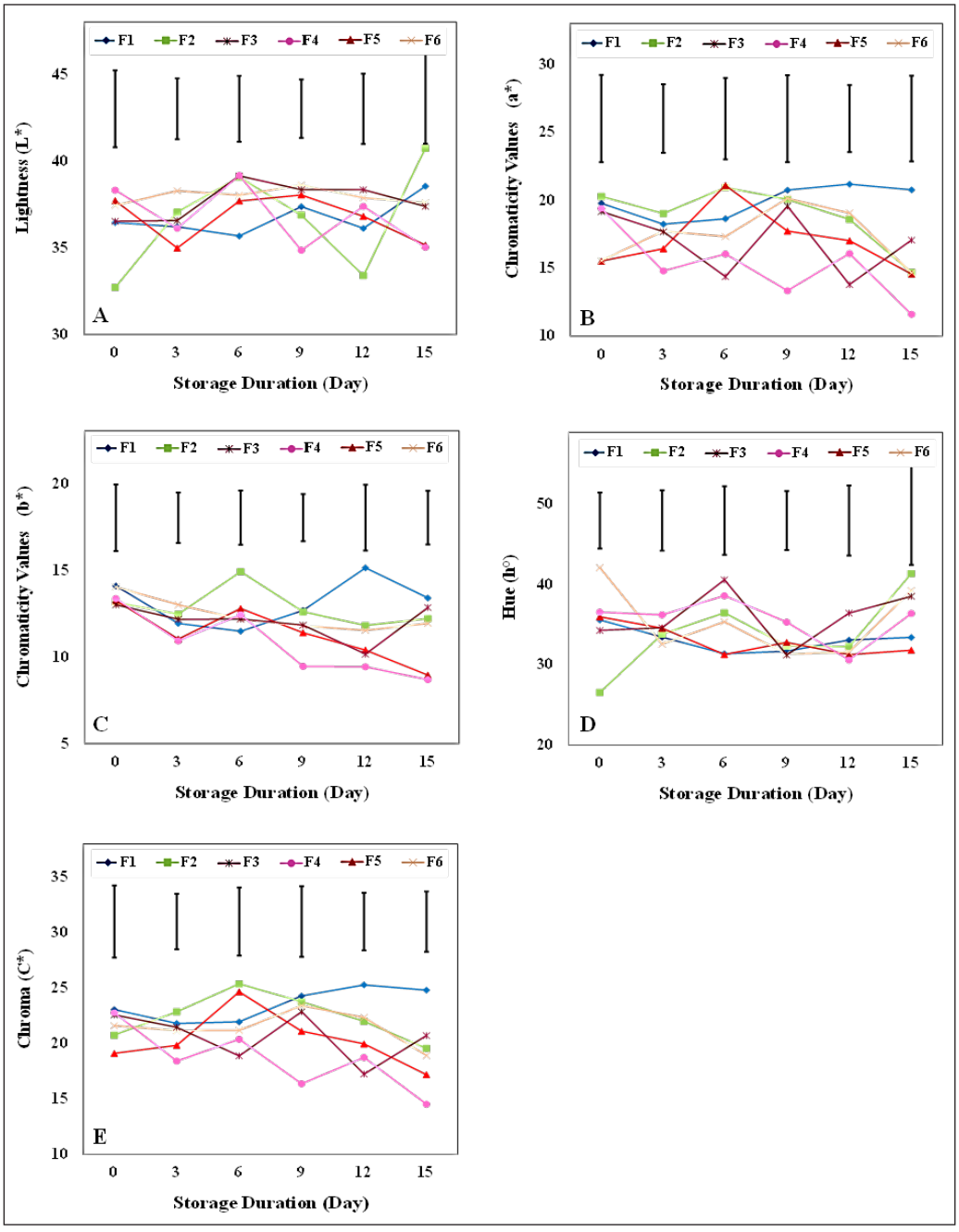


Figure 1. Effect of coating formulations on the colour of wax apples during 15 days of storage. A. lightness; B. chromaticity value a*; C. chromaticity value b*; D. hue angle; and E. chroma values. The vertical bar represents the HSD value at 5% significant level

better retention of the natural red hue of the fruit. For F4, stability was the worst, showing inconsistent colour retention. F4 experienced greater fluctuation of b* and C*, resulting in the least colour retention stability.

The enhanced colour retention in F3 and F6 can be explained by the presence of anthocyanins and polyphenols in the extract of roselle decoction residue. Natural anthocyanins are pigments that are water-soluble and capable of producing a wide range of colours from red to purple due to their ability to stabilise colour in response to structural changes that occur due to changes in pH (Wallace & Giusti, 2019). The presence of anthocyanins in edible coatings can protect coated fruit from colour degradation caused by oxidative or enzymatic activities, especially if the coating is protected by a physical barrier (Varasteh et al., 2012). The polyphenols in the roselle residue may also assist through antioxidant interactions that lessen the oxidative degradation of the pigments (Jung et al., 2022; Oren-Shamir, 2009). However, the present study did not quantitatively measure any enzymatic activities such as polyphenol oxidase (PPO).

As expected, the control (F1) exhibited the highest weight loss, followed by F2, F6, F5, F4, and F3 (Figure 2A) due to its high moisture content and delicate skin (Gunny et al., 2024; Kader, 2002). These factors make wax apples particularly susceptible to dehydration and mechanical damage. However, the roselle-based coatings significantly reduced this water loss compared to the control, which experienced nearly double the weight loss. This indicates that the coatings provided an effective semipermeable barrier to water vapour diffusion. Similar to weight loss, F3 and F4 stood out from all other formulations in preserving the firmness (Figure 2B). In other words, these two formulations were effective at preventing both weight loss and texture degradation. Meanwhile, the control (F1) exhibited the lowest firmness across all formulations throughout the 15-day storage period.

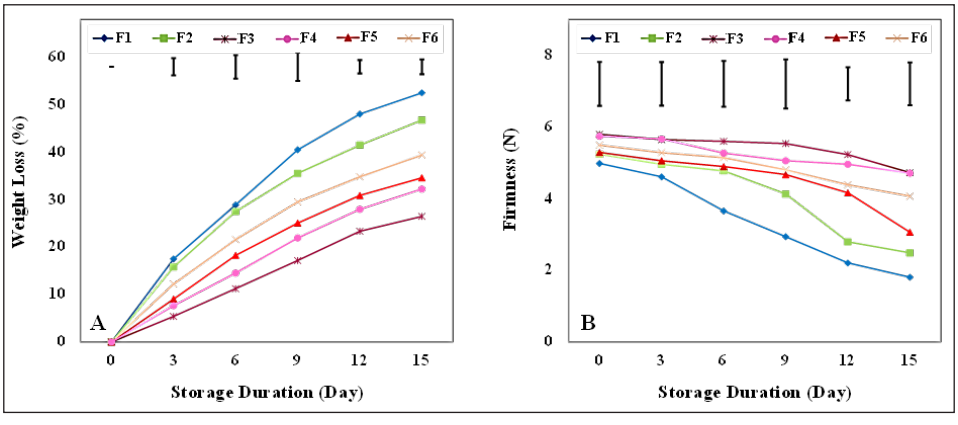


Figure 2. Effect of coating formulation on A. weight loss; and B. firmness of wax apples during 15 days of storage. The vertical bar represents the HSD value at 5% significant level

The softening of fruit during storage is closely linked to water loss and enzymatic degradation of cell wall components such as pectin, cellulose, and hemicellulose. In non-climacteric fruits like wax apples, ethylene production is inherently low, and there is no respiration spike postharvest (Shu et al., 2011). However, structural degradation still voccurs through the activity of enzymes such as polygalacturonase, pectin methyl esterase, and cellulase, which break down the middle lamella and weaken cell adhesion (Amit et al., 2017; Serrano & Valero, 2010).

The roselle-based coatings likely contributed to firmness retention through multiple mechanisms. First, the coatings serve as a semipermeable barrier that reduces moisture loss and may influence gas exchange, which is commonly associated with delayed softening in coated fruits. However, respiration rate was not directly measured in the present study. Second, polyphenols and organic acids in the roselle extract may contribute to the oxidative stability of cell wall components, although enzyme activity related to softening was not quantified. Third, the coating-forming components, particularly polysaccharides and mucilage (Sinha et al., 2022; Shinga & Fawole, 2023; Wang et al., 2020) from roselle residues, may provide an additional protective barrier to the fruit surface and minimise mechanical impact, thereby assisting in the retention of structural integrity of the fruit during storage.

Table 1 shows the effect of coating formulation on the development of fruit rot in wax apples over storage. Initially, the fruits appeared to be in good condition, exhibiting a glossy finish after being coated with the most effective roselle calyx residue extract from the *in vitro* bioassay. However, from day 12 onward, a noticeable decline in fruit quality and the onset of rot were observed in formulations containing roselle calyx residues, whereas other coatings began to show spoilage even earlier. The edible coatings certainly delayed the wax apples from fungal rot, but in the end, the coatings had little to no protective effect, signifying the need for the development of novel formulations in order to achieve better wax apple storage results.

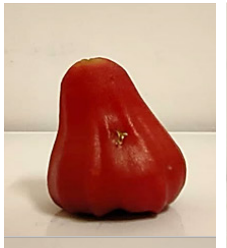
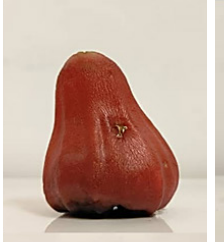
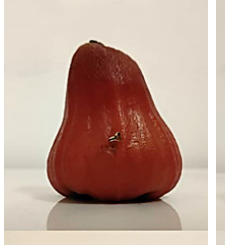
In the present study, the results demonstrated a significant interaction effect between coating formulation and storage duration (CF × SD) on SSC, TA, SSC/TA ratio, and pH values ($p \leq 0.001$). This indicates that the impact of storage duration on these quality attributes varied depending on the coating formulation, emphasising the role of edible coatings in modulating postharvest biochemical changes in wax apples.

Significant differences in SSC were observed across all storage days (Figure 3A). Throughout the storage durations, SSC exhibited an increasing trend, ranging from 5.00% on day 0, and rising up to 22.64% on day 15. During the early storage days, F3 showed a lower level of sweetness than that reported in F4 and F6. This indicates that the application of roselle-based coatings, especially formulations F3 and F5, effectively delayed the increase in SSC by limiting sugar metabolism and moisture loss. This effect, together with

Table 1
Effect of coating formulation on fruit rot of wax apples through storage duration

CF	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
F1						
F2						
F3						

Table 1 (*continued*)

CF	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
F4						
F5						
F6						

Note. CF: coating formulation; F1: negative control; F2: positive control; F3: fresh RC; F4: fresh DCR; F5: freeze-dried RC; F6: freeze-dried DCR; RC: roselle calyx; DCR: decocted cordial residue

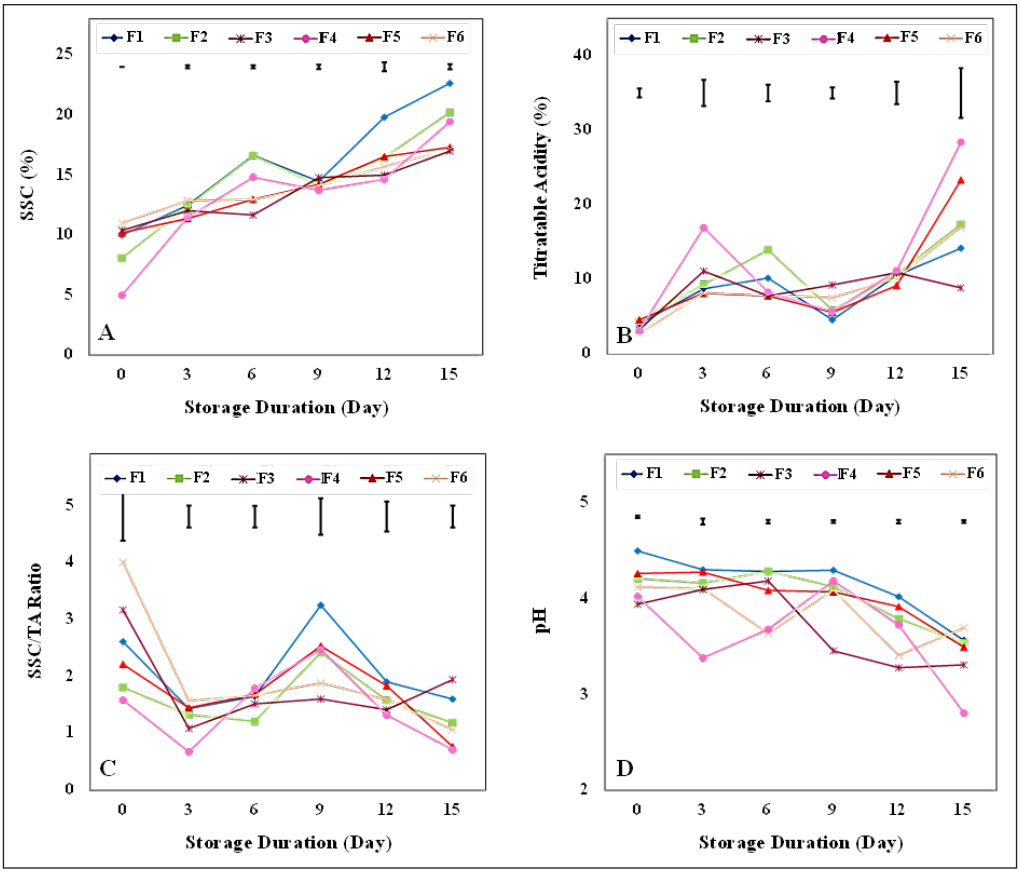


Figure 3. Effect of coating formulation on chemical quality of wax apples during 15 days of storage. A. soluble solid concentration (SSC); B. titratable acidity (TA); C. SSC/TA ratio; and D. pH. The vertical bar represents the HSD value at 5% significant level

their performance in preserving firmness, suggests their utility in extending postharvest quality and shelf life of wax apples by maintaining biochemical stability across multiple parameters. These findings are supported by similar studies in other fruits (Das et al., 2013; Gu et al., 2024; Sharma et al., 2019; Siti Fairuz, 2022).

In the present study, TA increased significantly throughout the 15-day storage period, except on day 12 (Figure 3B); meanwhile, pH values exhibited an inverse relationship with acidity, where lower pH values corresponded to higher acidity in wax apples (Figure 3D). Overall, a gradual decrease in pH was observed across all formulations, resulting in increased acidity throughout the storage period. Typically, organic acids such as citric and malic acid are consumed during ripening through respiration, leading to decreased TA and increased pH (Dávila-Aviña et al., 2011). However, in this study, TA increased while pH decreased, particularly in coated fruits. In non-climacteric fruits, organic acids

are typically consumed during respiration, resulting in stable or declining titratable acidity over storage. The deviation observed here is likely associated with the acidic nature of the roselle decoction residue extract. Roselle contains organic acids such as hibiscus acid, citric acid, and malic acid (Al-Wandawi, 2015; Izquierdo-Vega et al., 2020; Nur Amirah, 2015), which may have contributed to the measured acidity through surface interaction or limited diffusion into fruit tissue. However, migration was not directly quantified in this study. Additionally, the semipermeable coating may have reduced respiratory metabolism, thereby slowing endogenous acid utilisation. Collectively, these factors may explain the increase in TA observed in roselle-coated fruits.

As SSC increases and TA decreases over time, the SSC/TA ratio tends to rise, resulting in a sweeter taste. However, in the present study, the inverse was observed (Figure 3C). The decline in SSC/TA observed in coated fruits, especially F3 and F4, is likely attributed to the infusion of organic acids from the roselle extract into the fruit matrix, leading to a general decline in SSC/TA ratios over time. This change has significant impacts on the sensory qualities of fruits. As roselle is rich in hibiscus acid, malic acid, and citric acid (Al-Wandawi, 2015; Izquierdo-Vega et al., 2020), which can increase TA and decrease the relative contribution of SSC to the sweetness of the taste. This explains why even coated fruits with moderate SSC levels may taste very sour and lose their sweetness.

The interaction effect between coating formulation and storage duration was significant for vitamin C content, TPC, TFC, and DPPH radical scavenging activity ($p \leq 0.001$).

The presence of vitamin C, a highly sensitive compound, is easily exposed to oxidative degradation. It is especially susceptible to conditions involving high temperatures, the presence of oxygen, and alkaline, as well as metal ion conditions (Lee & Kader, 2000). However, in acidic conditions, its presence is considerably more stable as lower pH conditions slow the rate of oxidation. Considering this, coating formulations based on roselle may have enhanced the retention of vitamin C by creating a microenvironment that is slightly acidic around the surface of the fruit. The roselle calyx extracts contain some organic acids, which may help in lowering the pH and provide an antioxidant effect.

Of all the coating formulations, F6 demonstrated the highest efficacy in preserving vitamin C content throughout the storage duration, followed closely by F5 and F4 (Figure 4A). These findings signify the promising nature of the prospective coatings derived from roselle in shielding sensitive nutrients, including vitamin C, due to their combination of antioxidant components, acidic, and protective layers. With the consideration of SSC, TA, and pH, F6 and F5 are suggested to assist in the possible multiple layers of protective coatings and to extend the overall quality of postharvest wax apples. This was consistent with findings by Ali et al. (2010), in which it was shown that coated papayas had a much slower rate of vitamin C synthesis and degradation compared to the controls. Tigist et al. (2013) and Dávila-Aviña et al. (2014) also demonstrated that the coated tomatoes had slower increases

and eventual declines in vitamin C content during the storage period, which further confirms the nutrient retention in coated produce.

Among all coating formulations, F3 was found to retain more TPC than the control and was better able to retain phenolics over the storage period (Figure 4B). It should be noted that the Folin–Ciocalteu assay measures total reducing capacity and may respond to non-phenolic reducing substances such as sugars and ascorbic acid; therefore, TPC values are interpreted as comparative indicators rather than absolute phenolic quantification. All applied formulations, as well, experienced TPC reductions by day 3, which is in accordance with the process of oxidative stress and metabolic senescence. Although the roselle coatings still experienced TPC losses, they experienced the slowest losses. The TPC losses in phenolic evidence structures were likely due to oxidations, which are enzymes common during storage and ripening.

Such findings are consistent with earlier studies indicating a naturally occurring reduction of phenolic compounds during postharvest storage as a result of oxidative metabolism and stress from pathogens (Nur Atirah, 2023; Siti Fairuz, 2022). Furthermore, phenomena such as depletion of phenolic compounds and post-infection fungal accelerated infections (Lattanzio et al., 2006). The phenolic compounds are significantly consumed at the sites of infection when exposed to pathogens, which results in a significant decline in TPC over time.

The roselle decoction residue extract contains bioactive polyphenols and flavonoids, which are largely responsible for the antioxidant and antimicrobial activity of the edible coatings. When the coating is applied to the fruit, it creates a semipermeable layer that is expected to inhibit the diffusion of gases, particularly oxygen, which in turn may be able to slow down the oxidative degradation processes and preserve the phenolic compounds during storage. The organic acids of roselle may result in an increase in active acidity of the coating, which will reduce the activity of the enzyme responsible for browning. However, it should be noted that in this study, the PPO activity was not measured. Therefore, any assumptions made regarding the value of the suppression of the enzyme are purely speculative and require further validation of the enzyme. The coating's protective microenvironment played a significant role in maintaining TPC.

Furthermore, TFC retention in coated wax apples was significantly influenced by the relationship between storage duration and the specific coating applied (Figure 4C). With regard to the formulation used, F5 and F3 were the most promising as they demonstrated a moderate and consistent performance during the different storage periods. It is likely that the incorporation of the roselle extract altered the flavonoid biosynthetic pathway by activating the respective enzymes and providing oxidative protective walls against degradation. Furthermore, these observations reiterate the need for the development of edible coatings that preserve and refine active coatings to control and enhance the

biochemically active pathways of the coated fruits. This would potentially increase the functionality and nutritional value of the fruits after they have been harvested.

The observed increase in DPPH activity during storage may also reflect the fruit's biochemical defence response to postharvest stress, including pathogen exposure and oxidative conditions (Figure 4D). This phenomenon has been reported in other fruits like tomatoes and grapes, where increased antioxidant activity was linked to endogenous responses to microbial threats (Siti Fairuz, 2022; Wang et al., 2015). The application of roselle extract may have contributed to antioxidant enhancement through direct deposition of phenolic and flavonoid compounds onto the fruit surface. In addition, postharvest stress and pathogen exposure are known to influence endogenous antioxidant responses in fruits. However, the present study did not directly measure enzyme activity or gene expression associated with phenolic biosynthesis pathways. Therefore, the relative contribution of coating-derived antioxidants and fruit-induced biosynthesis cannot be definitively separated

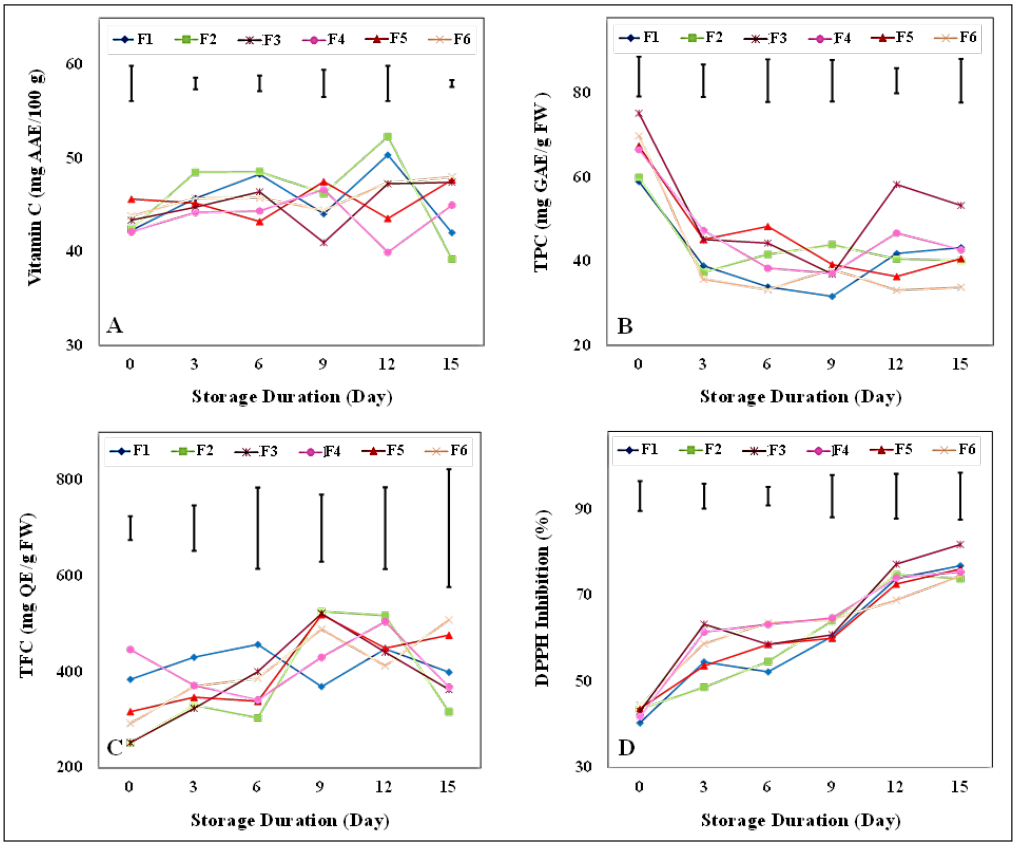


Figure 4. Effect of coating formulation on antioxidant activities of wax apples during 15 days of storage. A. vitamin C; B. total phenolic content; C. total flavonoid content; and D. DPPH inhibition. The vertical bar represents the HSD value at 5% significant levelV

within the scope of this study and requires further investigation. The elevated TPC and TFC observed in F3 and F5 are consistent with the enhanced DPPH activity in these treatments.

CONCLUSION

This study demonstrated that roselle decoction residue-based edible coatings improved the postharvest quality of wax apples during 15 days of ambient storage. Roselle-based coatings significantly enhanced colour retention, reduced weight loss, maintained firmness, and preserved key biochemical attributes, including titratable acidity, vitamin C, total phenolic content, total flavonoid content, and antioxidant activity, compared to uncoated controls. Among the formulations, fresh RC (F3) showed the most consistent performance across multiple quality parameters, followed by F6 and F4. Under wound-inoculation conditions, selected coatings also slowed down the growth of the *Pestalotiopsis* sp. These studies show that roselle decoction residues can be valorised as functional ingredients in edible coating systems for improving postharvest stability of wax apples.

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Behavioural Response of Cabbage White Butterfly (*Pieris rapae)* as Affected by Different Levels of Deltamethrin Insecticide

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ABSTRACT

The cabbage white butterfly (*Pieris rapae* L.) is a major pest of cruciferous crops, causing significant yield losses through larval feeding. Although synthetic pyrethroids such as deltamethrin are widely used for control, their efficacy may be compromised by larval behavioural responses that reduce lethal exposure. This study quantified the behavioural and toxic effects of deltamethrin on *P. rapae* larvae using a standardised one-way no-choice laboratory bioassay. The experiment was performed in a completely randomised design with three replications for each treatment under controlled conditions (20-23 °C; 30-60% relative humidity). Cabbage leaves were subjected to three coverage levels: water-treated control, 50% insecticide coverage, and 100% insecticide coverage at the recommended field rate (0.65 mL/L). Late-instar larvae were exposed to treated leaves. Larval survival, movement, and leaf area damage were recorded at multiple time points up to 72 hours. Complete insecticide coverage significantly reduced larval survival, locomotion, and feeding damage compared with partial coverage and controls, resulting in near-complete mortality by 72 hours. Partial coverage produced moderate but highly variable suppression, indicating inconsistent exposure. Early reductions in larval movement and feeding suggest avoidance of treated surfaces, while progressive declines over

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time indicate cumulative neurotoxic effects. These findings demonstrate that uniformity in insecticide coverage strongly influences both behavioural responses and control efficacy. Hence, optimising spray coverage is critical for effective *P. rapae* management and for reducing the risk of resistance development associated with sublethal exposure.

Keywords: Behavioural avoidance, deltamethrin, feeding deterrence, insecticide exposure, neurotoxic effects, *Pieris rapae*

INTRODUCTION

The cabbage white butterfly (*Pieris rapae* L.) is a globally significant pest of cruciferous crops, causing substantial economic losses through larval feeding on leaves and developing heads (Gautam et al., 2022). In the Philippines and across Southeast Asia, synthetic pyrethroids such as deltamethrin are widely used for *P. rapae* management due to their broad-spectrum efficacy, rapid action, and cost-effectiveness (Soltan et al., 2020). However, the long-term sustainability of pyrethroid-based control is threatened by the evolution of resistance, which can arise through physiological adaptations (e.g., target-site insensitivity, enhanced detoxification) and behavioural changes (e.g., avoidance of treated surfaces) (Haddi et al., 2018; Zalucki & Furlong, 2017).

According to Gómez-Guzmán et al. (2022), behavioural resistance is defined as the ability of insects to detect and avoid insecticide exposure before reaching lethal doses, thereby representing a critical but often overlooked mechanism of pest survival. Sublethal exposure to pyrethroids can induce altered feeding patterns, increased movement, or repellency, allowing insects to minimise contact with insecticides and survive (Muller et al., 2019; Nansen et al., 2016). For *P. rapae*, such behaviours may reduce the efficacy of foliar applications, particularly if larvae avoid treated leaves or exhibit reduced feeding. Notwithstanding its importance, few studies have quantitatively linked insecticide coverage levels to behavioural responses in *P. rapae* under controlled conditions.

Insecticide spray coverage in field conditions is often heterogeneous due to canopy structure, spray drift, and application inefficiencies. To approximate this variability under controlled laboratory conditions, a comparative assessment in this study was conducted between partial (50%) and full (100%) insecticide application coverage. This approach allows evaluation of larval behavioural responses under contrasting but field-relevant exposure scenarios. Hence, this study aimed to quantify the behavioural avoidance of *P. rapae* larvae in response to the different application coverage levels of deltamethrin on cabbage leaves, using a standardised "one-way no-choice" laboratory bioassay. Specifically, the experiment evaluated larval survival, movement, and leaf area damage in three experimental treatments: water-treated leaves (control), 50% insecticide coverage, and 100% insecticide coverage. It also addresses the study's limitations and recommended

directions for future research, including comparisons with resistant populations, dose-response assays, and field validation of laboratory observations.

METHODOLOGY

Study Area and Collection of Specimens

The experiment was conducted in November 2023 at the Entomology Laboratory, College of Agriculture, Benguet State University (BSU), La Trinidad, Benguet, Philippines. The experiment was conducted under consistent laboratory lighting (12-hour light: dark cycle) to ensure standardised conditions throughout the observation period. This is also to minimise circadian effects on larval feeding and movement. Late instar larvae of *Pieris rapae* were hand-collected from cabbage fields in the vicinity of BSU and transported to the laboratory, where they were contained in rectangular containers with mesh covers to ensure adequate ventilation. Cabbage leaves used in the bioassay were standardised by selecting similarly aged, fully expanded leaves from comparable canopy positions to minimise variation in nutritional content and leaf structure.

Experimental Design and Treatments

The experiment employed a "one-way no-choice" method (*Figure 1*), designed to simulate forced exposure scenarios common in dense crop plantings. The setup consisted of a dual-container arena connected by a sealed bridge, preventing larval escape while allowing movement between treated and untreated zones. Cabbage leaves were arranged on one side of the arena and treated according to the assigned treatment, while the opposite side remained untreated.

Figure 1. Experimental design using a "One-way no-choice method" in a dual-container setup to assess larval movement and feeding behaviour

This design allowed us to isolate the effects of insecticide coverage on larval behaviour, though it does not fully replicate field conditions where larvae may disperse or encounter heterogeneous spray patterns.

The same *Pieris rapae* larvae were observed across all time points (15, 30, and 60 minutes, and 2, 24, 48, and 72 hours) to monitor the progression of behavioural and toxic responses to deltamethrin exposure. Naïve larvae with no prior exposure to the insecticide or experimental conditions were used at the start of each replicate to standardise baseline conditions. Further, to ensure uniformity, larvae were introduced into the arenas 3-5 minutes post-treatment.

The bioassay was conducted under controlled ambient conditions (20-23°C, 30-60% relative humidity) following a Completely Randomised Design (CRD) with three replications per treatment. For each replicate, it contains two-late instar larvae housed in a shared container, and their responses (e.g., movement, survival, leaf damage) were recorded across all sampling time points. Meanwhile, despite a limited sample size per replicate, repeated measurements of the same larvae over time increased the robustness of the statistical analyses by capturing individual progression rather than cross-sectional comparisons. Treatments included T1 (water-treated control), T2 (50% insecticide coverage) and T3 (100% insecticide coverage, 0.65 mL/L deltamethrin, Decis 25 EC, Bayer).

Unfortunately, the use of late-instar larvae and a single recommended field rate of deltamethrin in this study may not capture the full range of behavioural or dose-dependent responses observed in earlier instars or at sublethal concentrations. Because this design focuses on immediate (short-term) and cumulative (long-term) responses to insecticide exposure, without confounding effects from repeated insecticide contact or prior experience.

Insecticide Application and Data Collection

Deltamethrin (Decis 25 EC, Bayer, 25 g/L active ingredient) was dissolved in 1 L of water at the manufacturer's recommended field rate (0.65 mL/L) using a beaker, then transferred to a 1.5-litre spray bottle. To avoid leaf saturation and ensure even coverage, a fine-nozzle atomiser was used for application. Two larvae were assigned to each treatment arena immediately after spraying.

Behavioural responses of the treated larvae per replicate were observed and recorded at 15, 30, and 60 minutes, and at 2, 24, 48, and 72 hours post-exposure. The observation intervals were strategically selected to capture both the immediate neurotoxic effects (e.g., hyperactivity, paralysis) and cumulative sublethal/lethal effects (e.g., feeding suppression, mortality) of deltamethrin on *Pieris rapae*, based on its known mode of action targeting insect sodium channels (Narahashi, 2000; Wolansky & Harrill, 2008) and documented residual toxicity in Lepidoptera (Desneux et al., 2007). Intermediate time points were excluded to minimise larval stress, reduce experimental complexity, and focus on the critical phases of insecticide response, acute symptoms and long-term outcomes,

while aligning with standard bioassay protocols for balancing biological relevance with practical feasibility (Leite et al., 2022; Wondafrash et al., 2012). The design used in this study ensures robust detection of treatment effects while adhering to ethical and logistical constraints.

Movement was measured objectively using pre-marked distances on the arena floor and standardised observation intervals to minimise observer bias during manual movement tracking. In addition, multiple observers cross-checked measurements to ensure consistency and reduce potential bias. The following parameters were measured:

i. Survival: Percent mortality was calculated using Abbott's corrected formula as shown below (Abbott, 1925; Fleming & Retnakaran, 1985). Though control mortality was negligible, Abbott's correction was applied as a standard practice.

   % Mortality (corrected) = [(% survival in control - % survival in treatment) / % survival in control] × 100

ii. Movement: The total distance travelled by each larva from the leaf centre was measured using marked reference points in the arena.
iii. Leaf Area Damage: Damage severity was scored using a modified categorical scale by Sorgini et al. (2019): 1 (sound): No damage, 2 (slight): 1-10 % damage, 3 (moderate): 11-20 % damage, 4 (heavy): 21-30 % damage and, 5 (severe): >31 % damage.

While linear displacement was used as a simplified measure of movement, future studies could employ video tracking to analyse path complexity and turning behaviour for a more nuanced understanding of larval responses.

Statistical Analysis

Leaf Area Damage (1-5 Ordinal Scale)

Kruskal-Wallis non-parametric ANOVA was performed to compare differences across treatments and time points, since the given nature of the leaf area damage ratings (1-5 scale) is ordinal. This statistical test was selected because it does not assume normality or equal intervals between ratings, making it appropriate for ordered categorical data (Siegel & Castellan, 1988). For post hoc analysis, Dwass-Steel-Critchlow-Fligner pairwise comparisons were used to control for multiple comparisons and identify specific treatment effects. Further, ordinal logistic regression was used to model the effect of treatment and time on damage ratings, as it accounts for the ordered nature of the response variable and provides odds ratios for interpreting treatment effects (McCullagh, 1980).

Larval Survival (Percentage Data)

Larval survival data were initially analysed using a two-way ANOVA after arcsine transformation, which is a common practice for proportional data to stabilise variance (Zar, 2010). However, Shapiro-Wilk tests indicated consistent deviations from normality ($p < 0.001$), and Levene's tests revealed heterogeneous variances ($p < 0.05$), even after transformation. These violations were likely due to the bounded nature of survival data (0-100%), small sample sizes ($n = 3$ per treatment/time combination), and zero-inflated values in high-dose treatments (e.g., 100% insecticide coverage). Consequently, Kruskal-Wallis non-parametric ANOVA was employed, followed by Dwass-Steel-Critchlow-Fligner pairwise comparisons, as non-parametric tests are robust to non-normality and heterogeneity of variance (Conover, 1999).

Distance Travelled (Continuous Data)

Shapiro-Wilk tests indicated non-normality ($p <0.001$), and Levene's tests confirmed heterogeneous variances ($p <0.05$). These violations were attributed to skewed distributions (e.g., clustering of lower values in high-dose treatments) and limited sample sizes, which constrained the robustness of parametric tests. Consequently, Kruskal-Wallis non-parametric ANOVA was also employed, followed by Dwass-Steel-Critchlow-Fligner pairwise comparisons to ensure valid inferences without relying on parametric assumptions (Dunn, 1964). Standard deviation (SD) was calculated to quantify within-group variability, while estimated marginal means were reported for treatment effects.

All analyses were conducted in jamovi (Version 2.6.13), which ensures both descriptive and inferential rigour.

Limitations of the Study

Several limitations must be acknowledged in this study. First, the focus on late-instar larvae and a single field rate of deltamethrin (0.65 mL/L) may not capture the full spectrum of instar-specific or dose-dependent responses, particularly in earlier, more susceptible stages (Zalucki & Furlong, 2017).

Second, the "one-way no-choice" design may be effective for isolating coverage effects, but it does not fully replicate field conditions, where larvae may disperse or encounter heterogeneous spray patterns. Likewise, the design does not disentangle contact toxicity from ingestion effects, as both routes were evaluated jointly.

Third, the sample size of two larvae per replicate (with three replicates per treatment) may limit statistical power for detecting subtle behavioural differences, although repeated measurements over 72 hours enhanced robustness.

Fourth, the study only focused on immediate and cumulative behavioural responses within 72 hours without assessing longer-term effects (e.g., recovery from sublethal exposure or delayed mortality).

Fifth, the laboratory setting fails to account for environmental factors (e.g., temperature, humidity) that could influence larval behaviour under natural conditions (Desneux et al., 2007).

Sixth, a solvent control was omitted, as the commercial formulation was compared to a water control, but future studies could include one to isolate formulation effects.

Finally, while insecticide degradation may occur over time, the progressive mortality and behavioural suppression observed suggest cumulative rather than diminishing effects. Hence, future research should incorporate physiological biomarkers, resistant strains, and field validation to further elucidate these mechanisms.

Ethical Considerations

This study adhered to standard entomological practices for insect bioassays, ensuring that all procedures were conducted with scientific rigour and ethical responsibility. *Pieris rapae* larvae were exposed to deltamethrin under controlled laboratory conditions, and prolonged exposure assays were terminated at 72 hours. After the experiment, surviving larvae were humanely euthanised via freezing at -20°C to prevent potential environmental contamination and align with standard entomological practices. No recovery assessment was conducted, as the study's objective was to evaluate the immediate and cumulative effects of insecticide exposure on larval survival, movement, and feeding behaviour. This approach aligns with guidelines for invertebrate research, which prioritise minimising unnecessary suffering while balancing scientific objectives.

RESULTS AND DISCUSSION

Larval Survival of *Pieris rapae* Under Varying Deltamethrin Insecticide Exposure

Table 1 presents the result of the mean larval survival of *Pieris rapae* exposed to three treatment levels, T1 (water control), T2 (50% deltamethrin coverage), and T3 (100% deltamethrin coverage), across seven-time intervals (0.25, 0.5, 1, 2, 24, 48, and 72 hours). Result revealed that the water control (T1) maintained consistently high survival (1.57 ± 0.00) across the first 24 hours, with a slight decline to 1.37 ± 0.196 at 48 and 72 hours. In contrast, T2 (50% coverage) showed no mortality (1.57 ± 0.00) during the first 2 hours, followed by a gradual decline to 1.37 ± 0.196 at 24 hours and 0.594 ± 0.373 at 48 and 72 hours, indicating partial but inconsistent suppression. Meanwhile, T3 (100% coverage) exhibited no mortality (1.57 ± 0.00) during the first hour, but survival dropped sharply to 0.592 ± 0.194 at 2 hours, ultimately reaching near-complete mortality (0.204 ± 0.194) by 72 hours, demonstrating the potent effect of full insecticide coverage.

Table 1
Mean (± SEM) larval survival, distance travelled (movement), and leaf area damage of pieris rapae *late-instar larvae exposed to varying deltamethrin coverage across seven time intervals*

Time Interval (hr)	Larval Survival			Distance Travelled (Movement)			Leaf Area Damage		
	T1	T2	T3	T1	T2	T3	T1	T2	T3
0.25	1.57 ± 0.00	1.57 ± 0.00	1.57 ± 0.00	43.8 ± 6.25	46.3 ± 2.98	36.9 ± 5.14	1.75 ± 0.250	1.75 ± 0.250	1.25 ± 0.250
0.50	1.57 ± 0.00	1.57 ± 0.00	1.57 ± 0.00	53.3 ± 3.33	41.9 ± 4.93	36.9 ± 5.14	1.75 ± 0.250	1.50 ± 0.289	1.25 ± 0.250
1	1.57 ± 0.00	1.57 ± 0.00	1.57 ± 0.00	64.0 ± 16.6	44.8 ± 3.77	36.9 ± 5.14	2.00 ± 0.00	1.75 ± 0.250	1.25 ± 0.250
2	1.57 ± 0.00	1.57 ± 0.00	0.592 ± 0.194	67.5 ± 21.0	36.3 ± 10.6	32.5 ± 11.4	2.00 ± 0.00	1.75 ± 0.250	1.25 ± 0.250
24	1.57 ± 0.00	1.37 ± 0.196	0.592 ± 0.194	69.3 ± 19.9	40.8 ± 13.9	32.5 ± 11.4	2.25 ± 0.250	2.00 ± 0.408	1.25 ± 0.250
48	1.37 ± 0.196	0.594 ± 0.373	0.592 ± 0.194	87.5 ± 37.5	35.6 ± 12.4	35.5 ± 11.9	2.25 ± 0.250	2.25 ± 0.629	1.25 ± 0.250
72	1.37 ± 0.196	0.594 ± 0.373	0.204 ± 0.194				2.25 ± 0.250	2.25 ± 0.629	1.25 ± 0.250

Statistical analysis using two-way ANOVA on arcsine-transformed larval survival data as shown in Table 2 revealed highly significant effects of treatment ($F_{2,63} = 21.14$, $p < 0.001$, $\eta^2 = 0.178$), time ($F_{6,63} = 14.41$, $p < 0.001$, $\eta^2 = 0.363$), and their interaction ($F_{12,63} = 3.85$, $p < 0.001$, $\eta^2 = 0.194$), explaining 17.8%, 36.3%, and 19.4% of the total variability, respectively. However, violated assumptions of homogeneity of variances (Levene's test, $p < 0.001$) and non-normality (Shapiro-Wilk, $p < 0.001$) necessitated the use of Kruskal-Wallis non-parametric ANOVA ($\chi^2_2 = 16.6$, $p < 0.001$, $\varepsilon^2 = 0.200$, Table 4), followed by Dwass-Steel-Critchlow-Fligner pairwise comparisons (Table 5). Post-hoc analyses confirmed that T3 significantly reduced larval survival compared to T1 ($p < 0.001$), while T2 did not differ significantly from T1 ($p = 0.139$) or T3 ($p = 0.082$), which highlights the critical role of complete coverage in achieving mortality (Table 3).

Further, the significant interaction effect ($\eta^2 = 0.194$) indicates that mortality dynamics vary over time, with T3s lethality becoming pronounced after 24 hours, likely due to cumulative toxicity and residual effects of deltamethrin (Leite et al., 2022). The immediate survival rates (15 minutes to 2 hours) were comparable across treatments, indicating that initial exposure to deltamethrin does not cause rapid mortality. However, survival diverged sharply from the control after 24 hours, particularly in T3, where full surface coverage and prolonged exposure led to cumulative toxicity, aligning with studies on plant-derived insecticides by Amongi (2016) and Viteri Jumbo et al. (2018).

The biological variability in T2 survival suggests that partial coverage may result in uneven insecticide distribution, allowing some larvae to avoid lethal exposure. This variability underscores the importance of consistent development (Desneux et al., 2007). While Abbott's correction was applied, its impact was minimal due to negligible control mortality, reinforcing the robustness of these trends.

To further elucidate mortality dynamics, time to event analyses (e.g., Kaplan-Meier survival curves) could be employed in future studies. Likewise, larger sample sizes and continuous survival metrics (e.g., video tracking) may improve statistical power and resolution.

Table 2
Two-way ANOVA results for arcsine-transformed percent larval survival of Pieris rapae *under varying deltamethrin coverage (T1: water control; T2: 50% coverage; T3: 100% coverage) across seven-time intervals (15 min–72 h)*

	Sum of Squares	df	Mean Square	F	p	η^2
Treatment	4.39	2	2.194	21.14	<.001*	0.178
Time	8.97	6	1.495	14.41	<.001*	0.363
Treatment ∗ Time	4.79	12	0.399	3.85	<.001*	0.194
Residuals	6.54	63	0.104			

Note. Significant differences ($p<0.05$) are indicated in asterisks (*). η^2 (eta-squared) represents the proportion of total variance in the dependent variable (e.g., distance travelled or survival) that is explained by the independent variable (e.g., treatment or time). Values range from 0 to 1, where 0.01 = small effect; 0.06 = medium effect; and ≥0.14 = large effect (Cohen, 1988)

Table 3
Tukey HSD post hoc comparisons of estimated marginal means for percent larval survival among treatments (T1: water control; T2: 50% deltamethrin coverage; T3: 100% deltamethrin coverage)

Comparison							
Treatment		Treatment	Mean Difference	SE	df	t	p_{tukey}
T1	-	T2	0.251	0.0861	63.0	2.92	0.013
	-	T3	0.559	0.0861	63.0	6.49	<.001*
T2	-	T3	0.308	0.0861	63.0	3.58	0.002*

Note. Comparisons are based on estimated marginal means. Significant differences ($p<0.05$) are indicated in asterisks (*)

Table 4
Kruskal-Wallis non-parametric ANOVA results for arcsine-transformed percent larval survival of Pieris rapae *under varying deltamethrin coverage*

	χ^2	df	p	ε^2
Larval Survival_Arcsine transformed	16.6	2	<.001*	0.200

Note. Significant differences ($p<0.05$) are indicated in asterisks (*). ε^2 (epsilon squared) represents the estimates of the proportion of variability in the ranked data

Table 5
Dwass-Steel-Critchlow-Fligner pairwise comparisons of percent larval survival among treatments (T1: water control; T2: 50% deltamethrin coverage; T3: 100% deltamethrin coverage)

		W	p
T1	T2	-2.69	0.139
T1	T3	-5.69	<.001*
T2	T3	-3.02	0.082

Note. Significant differences (p<0.05) are indicated in asterisks (*). 'W' represents the standardised difference between the rank sums of the two groups

Larval Movement Behaviour of *Pieris rapae* Under Varying Deltamethrin Insecticide Exposure

Larval distance travelled data is presented in Table 1, where movement behaviour of *Pieris rapae* was assessed across seven-time intervals (0.25-72 hours) under three treatments: T1 (water control), T2 (50% deltamethrin coverage), and T3 (100% deltamethrin coverage). Result revealed that T1 larvae exhibited the highest movement (e.g., 87.5 ± 37.5 mm at 48 hours), reflecting normal exploratory behaviour, while T3 larvae showed the lowest movement (e.g., 32.5 ± 11.4 mm at 24-48 hours), indicating strong behavioural suppression or neurotoxic effects. T2 larvae displayed intermediate movement (e.g., 40.8 ± 13.9 mm at 24 hours), which may demonstrate a dose-dependent response.

Statistical analyses were presented in Tables 6 and 7 for the two-way ANOVA, while Tables 8 & 9 were for the Kruskal-Wallis test. Results consistently confirmed that T1 larvae travelled significantly farther than T2 and T3 ($p < 0.05$), while T2 and T3 did not differ significantly ($p > 0.05$). This consistency between parametric and non-parametric results reinforces the reliability of the findings, highlighting that full insecticide coverage (T3) most effectively suppresses larval movement, likely due to combined behavioural avoidance and neurotoxic impairment.

In this study, behavioural resistance is operationally defined as the adaptive avoidance of deltamethrin-treated surfaces by *Pieris rapae* larvae, manifesting as reduced locomotor activity or repellency in response to chemical cues (Foster et al., 2007; Haynes, 1988; Hubbard & Murillo, 2024). Meanwhile, neurotoxic impairment refers to the physiologically mediated inhibition of locomotion or feeding, arising from deltamethrin's disruptive effects on neural function (Desneux et al., 2007; Wolansky & Harrill, 2008).

Observations obtained in the bioassay reveal a distinct temporal and dose-dependent pattern in larval movement: larvae in the control group (T1) exhibited highly variable and extensive displacement (e.g., 64.0 ± 16.6 mm at 1 hour) at early time points, reflecting uninhibited exploratory and foraging behaviour characteristic of undisturbed *P. rapae* (Tsuji et al., 2018). This absence of insecticide stress in T1 permitted natural locomotor activity, consistent with baseline observations in lepidopteran larvae.

Table 6
Two-way ANOVA results for distance travelled by Pieris rapae *larvae under varying deltamethrin coverage (T1: water control; T2: 50% coverage; T3: 100% coverage) across six-time intervals*

	Sum of Squares	df	Mean Square	F	p	η^2
Treatment	11192	2	5596	6.734	0.002*	0.184
Time	837	5	167	0.202	0.960	0.014
Treatment ∗ Time	4016	10	402	0.483	0.894	0.066
Residuals	44875	54	831			

Note. Significant differences ($p<0.05$) are indicated in asterisks (*). η^2 (eta-squared) represents the proportion of total variance in the dependent variable (e.g., distance traveled or survival) that is explained by the independent variable (e.g., treatment or time). Values range from 0 to 1, where 0.01 = small effect; 0.06 = medium effect; and ≥0.14 = large effect (Cohen, 1988)

Table 7
Tukey HSD post hoc comparisons of estimated marginal means for distance travelled by Pieris rapae *larvae among treatments (T1: water control; T2: 50% deltamethrin coverage; T3: 100% deltamethrin coverage)*

Treatment		Treatment	Mean Difference	SE	df	t	p_{tukey}
T1	-	T2	23.31	8.37	54.0	2.785	0.020
	-	T3	29.03	8.37	54.0	3.470	0.003*
T2	-	T3	5.73	8.32	54.0	0.688	0.771

Note. Comparisons are based on estimated marginal means. Significant differences ($p<0.05$) are indicated in asterisks (*)

Table 8
Kruskal-Wallis non-parametric ANOVA results for distance travelled by Pieris rapae *larvae under varying deltamethrin coverage*

	χ^2	df	p	ε^2
Distance travelled by the larvae	16.4	2	<.001*	0.231

Note. Significant differences ($p<0.05$) are indicated in asterisks (*). ε^2 (epsilon squared) represents the estimates of the proportion of variability in the ranked data

Table 9
Dwass-Steel-Critchlow-Fligner pairwise comparisons of distance travelled by Pieris rapae *larvae among treatments (T1: water control; T2: 50% deltamethrin coverage; T3: 100% deltamethrin coverage)*

		W	p
T1	T2	-3.68	0.025
T1	T3	-5.49	<.001*
T2	T3	-2.47	0.188

Note. Significant differences ($p<0.05$) are indicated in asterisks (*). 'W' represents the standardised difference between the rank sums of the two groups

In contrast, larvae exposed 100 % deltamethrin coverage (T3) displayed minimal movement (e.g., 32.5 ± 11.4 mm at 24 hours) and low variability, indicating strong behavioural avoidance and/or neurotoxic impairment. The reduced variability observed under full insecticide coverage (T3) reflects biologically constrained responses near zero movement and feeding rather than statistical compression. Such patterns are consistent with strong toxic or deterrent effects limiting behavioural expression.

Meanwhile, the intermediate movement (e.g., 40.8 ± 13.9 mm at 24 hours) in T2 (50% coverage), where larvae travelled farther than in T3 but significantly less than in T1, supports a dose-dependent response, consistent with findings by Wondafrash et al. (2012). Such behaviour is underpinned by their sensory capacity to detect volatile and contact chemical cues from both host plants and insecticides (Miles et al., 2005; Tsuji et al., 2018).

Understanding the dual mechanisms of behavioural resistance and neurotoxic impairment can guide the development of more effective insecticide application strategies, ensuring both immediate deterrence and sustained larval suppression in integrated pest management (IPM) programs. The suppressed movement in T3 has critical implications for pest management, as reduced larval dispersal can limit both direct damage and the spread of infestations.

Future studies could employ video tracking to analyse path complexity, turning behaviour, and velocity, offering deeper insights into the mechanisms of avoidance and impairment (Charreton et al., 2015). This would enhance our understanding of behavioural resistance and neurotoxic effects, guiding the development of more effective insecticide application strategies in IPM programs.

Effect of Insecticide Coverage on *Pieris rapae* Feeding Damage

Mean leaf area damage is presented in Table 1, wherein the effect of deltamethrin coverage on *Pieris rapae* feeding behaviour was assessed by measuring mean leaf area damage across seven-time intervals (0.25-72 hours) under three treatments: T1 (water control), T2 (50% coverage), and T3 (100% coverage). Result revealed a clear treatment-dependent suppression where T1 larvae (control) caused increasing damage over time, with mean ratings rising from 1.75 ± 0.25 at 0.25 hours to 2.25 ± 0.25 at 24-72 hours, reflecting unrestricted feeding. In contrast, T3 larvae (100% coverage) showed minimal and consistent damage (mean = 1.25 ± 0.25 across all time points), indicating near-complete feeding suppression. T2 larvae (50% coverage) exhibited intermediate damage, with ratings increasing from 1.75 ± 0.25 at 0.25 hours to 2.25 ± 0.63 at 48-72 hours, suggesting partial but inconsistent feeding deterrence.

Statistical analyses confirmed these patterns as shown in Table 10, where Kruskal-Wallis ANOVA revealed a highly significant effect of treatment on leaf area damage ($\chi^2_2 = 27.6$, $p < 0.001$, $\varepsilon^2 = 0.332$), explaining 33.2% of total variability.

Table 10
Kruskal-Wallis non-parametric ANOVA results for leaf area damage ratings (1–5 scale) caused by Pieris rapae *larvae under varying deltamethrin coverage (T1: water control; T2: 50% coverage; T3: 100% coverage)*

	χ^2	df	p	ε^2
Damage_Scale	27.6	2	<.001*	0.332

Note. Significant differences (p<0.05) are indicated in asterisks (*). ε^2 (epsilon squared) represents the estimates of the proportion of variability in the ranked data

Table 11
Dwass-Steel-Critchlow-Fligner pairwise comparisons of leaf area damage ratings (1–5 scale) among treatments (T1: water control; T2: 50% deltamethrin coverage; T3: 100% deltamethrin coverage)

		W	p
T1	T2	-2.10	0.297
T1	T3	-7.27	<.001*
T2	T3	-5.04	0.001*

Note. Significant differences (p<0.05) are indicated in asterisks (*). 'W' represents the standardised difference between the rank sums of the two groups

Table 12
Ordinal logistic regression model fit measures for leaf area damage ratings (1–5 scale) caused by Pieris rapae *larvae, modelling the effects of treatment and time.*

Model	Deviance	AIC	R^2_{McF}
1	122	134	0.214

Note. Models estimated using a sample size of N=84. The dependent variable 'Damage_Scale' has the following order: 1 | 2 | 3 | 4

Table 13
Ordinal logistic regression coefficients for leaf area damage ratings (1–5 scale) caused by Pieris rapae *larvae, modelling the effects of treatment and time*

Predictor	Estimate	SE	Z	p
Time	0.0163	0.00950	1.72	0.085
Treatment:				
T2 – T1	-0.9117	0.62624	-1.46	0.145
T3 – T1	-3.2810	0.68904	-4.76	<.001*

Note. Significant differences (p < 0.05) are indicated in asterisks (*)

Pairwise comparisons (Dwass-Steel-Critchlow-Fligner, Table 11) showed that T3 caused significantly lower damage than T1 ($p < 0.001$) and T2 ($p = 0.001$), while T1 and T2 did not differ ($p = 0.297$). Ordinal logistic regression further supported these results (Tables 12-13), with T3 significantly reducing damage compared to T1 ($p < 0.001$) and no significant effect of time ($p = 0.085$) or T2 ($p = 0.145$). The large effect size ($\varepsilon^2 = 0.332$) and moderate McFadden's R^2 (0.214) underscore the biological significance of full coverage (T3) in suppressing feeding, while partial coverage (T2) failed to reduce damage relative to controls.

The low variability in T3 suggests a uniform larval response, likely due to high mortality or deterrence, whereas the higher and more variable damage in T1 and T2 reflects inconsistent larval behaviour and uneven exposure to untreated leaf areas. This aligns with the studies of Desneux et al. (2007) and Nansen et al. (2016), highlighting that suboptimal coverage leaves gaps in protection, leading to variable and suboptimal pest control outcomes.

These results further highlight the critical need for uniform insecticide application, as suboptimal coverage (T2) leads to inconsistent feeding suppression and risks accelerated resistance development due to sublethal exposure (Desneux et al., 2007; Nansen et al., 2016).

Despite the focus on *P. rapae* in this study, the behavioural responses to pyrethroids, such as avoidance and locomotor inhibition, are likely conserved across related crucifer-feeding Lepidoptera (Haynes, 1988). Hence, future work should prioritise larger sample sizes and continuous damage metrics (e.g., digital image analysis) to address distributional challenges and refine these findings.

CONCLUSION

The result of this study demonstrates that insecticide coverage level strongly influences both the behavioural and toxic responses of *Pieris rapae* larvae to deltamethrin. Complete coverage at the recommended field rate resulted in near-total mortality, minimal larval movement, and consistently low feeding damage. This implies an effective suppression through combined behavioural avoidance and cumulative neurotoxic effects. In contrast, partial coverage produced variable outcomes, with moderate reductions in survival and feeding that were inconsistent across replicates, highlighting the limitations of incomplete exposure.

The result also suggests that behavioural responses contribute to early avoidance of treated leaf surfaces, as shown in the rapid suppression of larval movement and feeding shortly after exposure. Conversely, the sustained declines in activity and survival of *P. rapae* larvae over time reflect progressive toxic effects; thus, both behavioural avoidance and toxicity contribute to insecticide efficacy and should be interpreted as potential

precursors to resistance development under prolonged field selection pressure rather than direct evidence of resistance. Hence, these findings emphasise that suboptimal insecticide coverage can reduce control reliability and potentially promote resistance by allowing survival under sublethal exposure.

This study underscores the importance of uniform insecticide application for effective *P. rapae* management. Incorporating behavioural assessments into insecticide evaluation can improve understanding of control failures and guide the development of more sustainable pest management strategies. Future research should expand on these findings by examining dose-response relationships, resistance populations, and field-scale validation to better integrate behavioural dynamics into integrated pest management programs.

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DISCLAIMER

AI tools were not employed during the preparation of this manuscript; particularly, the core intellectual contributions, including study design, data collection, analysis, interpretation of results, and conclusions, were independently developed and validated by the authors. The authors take full responsibility for the accuracy, originality, and integrity of the manuscript and confirm that AI functioned solely as a supportive language correction aid rather than a source of original scientific content for improved clarity and enhanced readability. In addition, any AI-assisted vocabulary in the manuscript was critically reviewed, selectively adapted, and integrated by the authors to ensure that the wording accurately reflects the intended scientific meaning and disciplinary context.

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Storage Reserve Composition and Storage Behaviour of *Amorphophallus muelleri* Blume Seed

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ABSTRACT

Amorphophallus muelleri Blume, belonging to the Araceae family, has a high economic value due to the glucomannan content used in both food and non-food industries. Propagation of *A. muelleri* through seed is more efficient than through bulbs and corms because the seed offers higher multiplication and bulking rates. However, limited information on the seed composition and storage behaviour poses challenges for handling and storage. Therefore, this study aimed to determine the seed chemical composition, classify the storage behaviour, and assess the effect of storage temperature on viability. The results showed that the seed contained 51% starch as the primary reserve compound and 15.74% glucomannan. Rapid drying at 35 °C with 40% relative humidity (RH) was found lethal to the seed, while slow drying at 22 °C with 10% RH was harmless for viability. Seed preserved the viability (78-91 %) when the moisture content remained above 50%, but lost viability when the moisture content reduced slowly to 30.9% (MDN1), 21.3 % (MDN2), and 43.25% (PWTA), showing that *A. muelleri* seed is recalcitrant. Moisture content of 60.2% (L1), 62.9% (L2), and 60.2% (L3) led to loss of seed viability with germination average of 5 % during the third month at refrigerator storage (5-7 °C, 40-50 % RH). In contrast, controlled temperature storage of 27-32 °C (>60% RH) maintained seed quality, including water content above 48% and germination above 80% during five months, and for three months.

Keywords: Glucomannan, moisture content, recalcitrant, seed desiccation, starch, viability

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INTRODUCTION

Amorphophallus spp. are among the most widely grown Araceae species in tropical and subtropical regions, including

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Indonesia. More specifically, *Amorphophallus muelleri* Blume; sin. *A. oncophyllus* Prain. is highly valued due to the glucomannan content, which is used in both food and non-food industries (Kurniawan et al., 2011). Based on the bulking and multiplication rate, *A. muelleri* propagation using seed is the most effective method compared to bulb and corm. In contrast to bulbs and corms, which require about 300 kg/ha and 1500 kg/ha of planting material, respectively, botanical seed propagation requires only 30-40 kg per ha.

Seed longevity refers to the period during which the seed remains viable and survives while in storage or under natural conditions until germination in the field and production of a normal plant. It is influenced by seed moisture content, storage conditions, temperature, and relative humidity (RH), with the effect varying among species (Nadarajan et al., 2023; Pirredda et al., 2024). Therefore, classification of seed storage behaviour is required to provide appropriate storage methods (Hong et al., 1996). There are three categories for seed storage behaviour, including orthodox, recalcitrant, and intermediate. Orthodox seed tolerates either desiccation (moisture content of 10 %) or sub-zero temperatures without damage, while recalcitrant and intermediate seed are sensitive to desiccation and chilling. Recalcitrant seeds do not survive in dry conditions or low moisture content. This implies that the seeds are viable only for a limited period and cannot withstand moderate and long-term storage. Although less resilient compared to orthodox, intermediate seed can withstand higher degrees of desiccation than recalcitrant seed (Hay et al., 2022; Pirredda et al., 2024; Tchokponhoué et al., 2019). *A. muelleri* is reportedly recalcitrant based on the high moisture content of harvested seed (Sari et al., 2019). However, there is limited evidence regarding the seed tolerance to desiccation and the optimum range of moisture to preserve the viability.

An optimum environment, including temperature and RH, is needed to preserve seed viability during storage. Temperature is one of the crucial factors preventing seed deterioration during storage. The optimum temperature can be determined based on the character of the seed. Orthodox seed requires low temperature and humidity storage conditions, while recalcitrant seed is sensitive to low temperature. For recalcitrant seed, identifying the lowest temperature that can be tolerated without inducing chilling injury is essential to minimise metabolic activity and prolong storage life. Studies on recalcitrant seed storage showed that temperature and period varied across species. For example, *Araucaria angustifolia* stored at 5 °C maintained high viability for up to 12 months and then decreased in 19 months (Gasparin et al., 2020). Environmental conditions also influenced the storage of *Ekebergia capensis*. Seed from the temperate zone maintained viability above 80% after 12 weeks of storage at 1 °C, 3 °C, and 6 °C. In contrast, seed from the sub- tropical zone retained viability of 100% at 6 °C and 16 °C, but lost viability after 38 d at 3 °C. Seed from the tropical zone lost viability at 3 °C (after 9 days) and decreased to 10% and 35% viability at 6 °C and 16 °C, respectively (Bharutha et al., 2020).

Lipids, carbohydrates, and proteins are the main compounds of seeds, and the relative proportions vary among species. Carbohydrates are a major energy reserve and serve as an energy source during seed germination. Compared to lipids, carbohydrates are relatively stable and resistant to oxidation. Therefore, seeds with higher starch content generally have better longevity (Hay et al., 2022). Lipids, specifically those in seeds with high oil content, are stored as energy reserves and are essential for metabolism during germination. However, seeds with high lipid content tend to have shorter longevity compared to starchy ones. This is because lipid or oil content affects the amount of water absorption at a given RH. Lipids are prone to oxidation and changes in fatty acid content during storage, which accelerates damage (Rao et al., 2023). Proteins in seeds function as enzymes, storage proteins, and structural components. During germination, proteins are broken down to provide amino acids for seedling development. This degradation can affect seed vigour and viability due to susceptibility to denaturation, oxidation, and environmental stress, reducing seed life (Shibata et al., 2020).

Limited information on storage reserve composition and storage Behaviour of *A. muelleri* seed has become an obstacle for handling and conservation. Therefore, this study aimed to (i) determine compounds that play important roles in seed handling and conservation, (ii) classify the category of seed storage behaviour by observing the effect of desiccation on moisture content and viability, and (iii) identify the effect of the environment on viability during five months of storage.

MATERIALS AND METHODS

Experiment 1: Determination of Storage Reserves Composition: Starch, Protein, Lipid, and Glucomannan

A single seed lot of *A. muelleri* var Madiun 1 was harvested in June 2022 in Madiun Regency and used to determine seed reserve composition, including starch, protein, lipid, and glucomannan. Total starch seed was measured on a wet weight basis. Samples of 0.5 g seed were ground and dissolved in 2 ml Aquadest, then incubated at 100 °C for 15 minutes. About 5 ml of perchloric acid (9.2N) was added when the suspension was cooled and incubated for 15 minutes. Water was added until the volume of the final suspension reached 10 ml, followed by centrifugation to separate the supernatant and sediment. The supernatant was taken and transferred to another container. The sediment was added with 2.5 ml of perchloric acid of 4.6 N and incubated for 15 minutes. Water was added until the volume of the final suspension reached 10 ml, and the suspension was centrifuged to separate the supernatant and sediment. A 1 ml sample was taken, and 10 ml of anthrone 0.1 % was added, followed by boiling in a water bath for 7.5 minutes. The starch concentration was determined using a spectrometer at ƛ 630 nm, and calculated using a standard curve, constructed with glucose as the standard (Qalsum et al., 2015).

The crude protein content of the seed sample was determined using the Kjeldahl method of AOAC, while crude fat content was measured by extracting the fat using the Soxhlet method. For glucomannan determination, the seed sample was washed and cut into 2-3 mm pieces, then soaked with 1 % (w/v) sodium bisulfite for 1 minute. Subsequently, the sample was dried at 120 °C for 40 minutes and continued at 60 °C until it reached constant weight, followed by grinding into fine flour (Chua et al., 2012). Glucomannan content was analysed using the gravimetry method, measured on a dry basis. A 5-gramme sample was weighed and dissolved in 50 ml of warm water at 75 °C, with 0.5 grammes of aluminium sulfate added, followed by stirring for 35 minutes using a shaker. The sediment was removed from the solvent by centrifugation at 2000 rpm for 30 minutes. The supernatant was collected and added to isopropyl alcohol in a 1:1 ratio while stirring until a clot formed. The sample was filtered using filter paper, and the residue/clot was dried at 60 °C for 24 hours, then weighed.

Experiment 2: Effect of Desiccation on Seed Moisture Content and Viability

Different desiccation methods to reduce seed moisture content may affect viability. Therefore, various types of desiccation methods need to be evaluated to assess the effect on the reduction of viability. Seed desiccation tolerance must be determined to classify the *A. muelleri* category of storage behaviour. Based on the protocol from Hong and Ellis (1996), seed should dry to 10-12% moisture content to determine viability. Seed are classified as orthodox or intermediate when most survive at this moisture level, while seed are considered recalcitrant when most lose viability.

Three seed lots of *A. muelleri* Blume var. Madiun 1 from Central Java, Indonesia were used for experiment 2. MDN1 and MDN2 were harvested in Madiun Regency from June, 9-11th 2022, and June, 6-9th 2022, respectively, while PWTA was harvested in Purwokerto Regency from June, 22-24th. This experiment was conducted from August 2022 until February 2023. Before and after the desiccation test, seed lots were tested for moisture content and germination. The moisture content was tested using the low-temperature method at 103 °C for 17 h and reported as a percentage of fresh weight.

Experiment 2 was performed using a randomised complete design with two factors, namely seed lots (MDN1, MDN2, PWTA) and rate of seed desiccation. Three different rates of desiccation test were designed, namely rapid, moderate, and slow. To reduce seed moisture content, 250 g of seed were evenly spread on trays and subjected to three different desiccation conditions: (i) rapid desiccation using a dehydrator with warm free airflow at 35 °C and 40% RH, (ii) moderate desiccation in an incubator at 35 °C and 50% RH, and (iii) slow desiccation using silica gel placed in a sealed plastic box beneath the samples at 22 °C and 10% RH. The temperature and RH were measured by using a thermohygrometer. To estimate the reduction of seed moisture content, the samples were weighed regularly

and calculated using the following Equation 1 (International Seed Testing Association [ISTA], 2021):

$$\text{Weight of seed lot at a certain \% MC} = (\text{initial weight}) \times [(100-\text{initial MC}) / (100-\text{desired MC})] \quad [1]$$

where MC: Moisture content

Experiment 3: Effects of Storage Environment on Seed Germination

Three lots of *A. muelleri* Blume var Madiun 2 (L1, L2, L3) were used to ascertain the optimum environment for storing seed. L1 was harvested from Klangon Regency (East Java) on 17th July 2023, while L2 and L3 were harvested from Tapos Regency (West Java) on 10th and 25th July 2023, respectively, with 60-63 % moisture content. The experiment was organised using a nested completely randomised design of two factors and three replications, namely storage environment and periods.

About 300 g of seed sample was stored in three different environments of (S1) ambient temperature at 27-32 °C (>60% RH), (S2) controlled temperature at 20-23 °C (50-60% RH), and (S3) refrigerator at 5-7 °C (40- 50% RH) during 5 months (M0-M5). The temperature and RH were measured by using a thermohygrometer. Seed samples were stored in paper bags and tested for moisture content, germination, and T_{50} for each month. The moisture content was tested using the low-temperature method at 103 °C for 17 h and reported as a percentage of fresh weight.

Seed Moisture Content Determination

Two replicates, which were drawn from a seed sample, were cut into pieces no thicker than 7 mm for a maximum of 4 minutes. Each replicate was weighed to four decimal places for 4.5-5 g. Subsequently, containers and covers (porcelain cups) were weighed before and after filling with the sample. The containers were then placed quickly next to the cover in an oven maintained at 103 °C for 17 h. After heating, it cooled down in a desiccator for 30 minutes. The containers with the cover were weighed, and the moisture content was calculated using Equation 2 (ISTA, 2021).

$$\text{Seed MC (\%)} = [(M2-M3) / (M2-M1)] \times 100\% \quad [2]$$

where: M1: The weight of the container and cover (g)
M2: The weight of the container, cover, and content before drying (g)
M3: The weight of the container, cover, and content after drying (g)

Germination Test

Three replicates of 50 seeds were sown, using the between-paper method, and incubated at 25 °C. The germination test results are expressed as the percentage by number of normal seedlings (ISTA, 2021). Total germination was calculated using Equation 3:

$$\text{Germination (\%)} = (\text{number of normal seedlings/number of seed}) \times 100 \quad [3]$$

Seedling evaluation was conducted at three-day intervals until germination reached 50% of the final germination value (T_{50}). The germination period was recorded until the seed sample reached the maximum rate of T50 calculated using Equation 4:

$$T50 = Ti + (((N+1/2)-Ni)\ (Tj-Ti))/(Nj-Ni) \quad [4]$$

where T50 is the median germination time, N is the final number of germinated seed, while Ni and Nj are the total number of seed germinated in adjacent counts at time Ti and Tj, respectively, when $Ni < ((N+1)/2) < Nj$ (Kumar et al., 2021).

Normal seedlings were defined as intact seedlings that had all healthy essential structures, including a shoot and root system. The cotyledon types (bifacial hypophyll and unifacial hyperphyll) and the presence or absence of endosperm were used to categorise araceous seedlings. *A. muelleri* had a large, green storage organ of cotyledonary hyperphyll, with no or only inconspicuous traces of endosperm. Before the leaf appeared, it had one or more cataphylls. The first shoot-born root pierced the cotyledon body at the base of the shoot. The hypocotyl and primary roots were absent. It had one or several cataphylls before the leaf appeared. Hypocotyl and primary roots were missing, then the first shoot-born roots broke through the body of the cotyledon at the base of the shoot (Figure 1) (Fadhilah et al., 2025; Tillich, 2014).

Abnormal seedlings showed rot symptoms due to primary infection. The internal condition of the ungerminated seed was assessed by lightly pushing with the thumb. Seeds were classified as dead when fungal growth was visible or when internal tissues leaked or appeared rotten (Figure 2).

Data Analysis

The effect of desiccation on seed moisture content and viability was determined by decreasing the level of moisture content and germination. Seed desiccation tolerance was determined by the lowest moisture content at which either the seed was preserved or lost its viability. Seeds were determined orthodox or intermediate when the germination rate was more than 50 % with 10-12 % moisture content. On the other hand, the seed was determined to be recalcitrant when the germination rate was less than 50 % with 10-12 % moisture content.

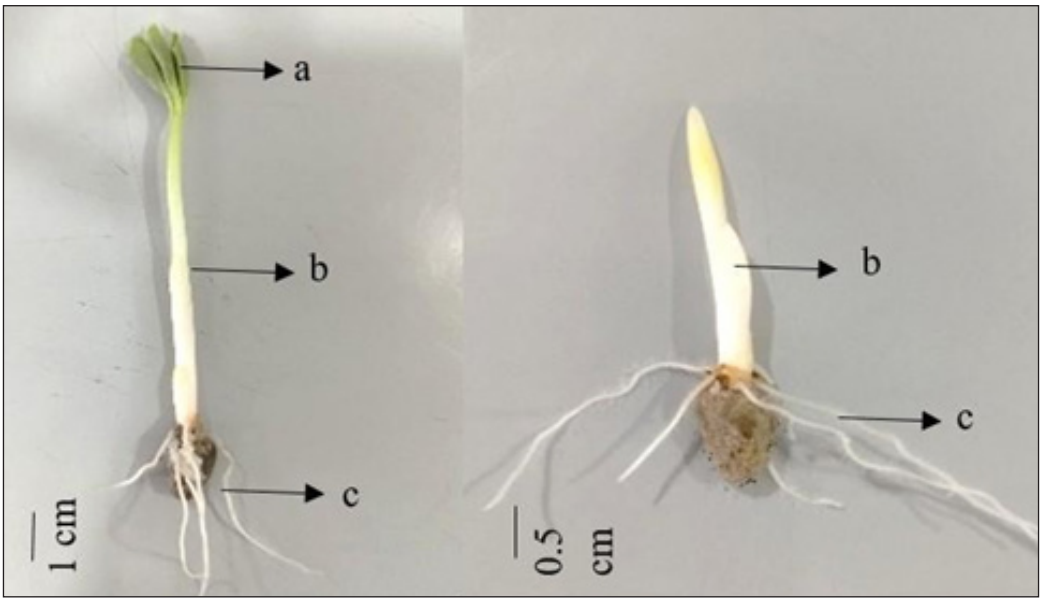

Figure 1. The normal seedling structures of *A. muelleri* (a) leaf; (b) cataphyll; (c) shoot-borne root

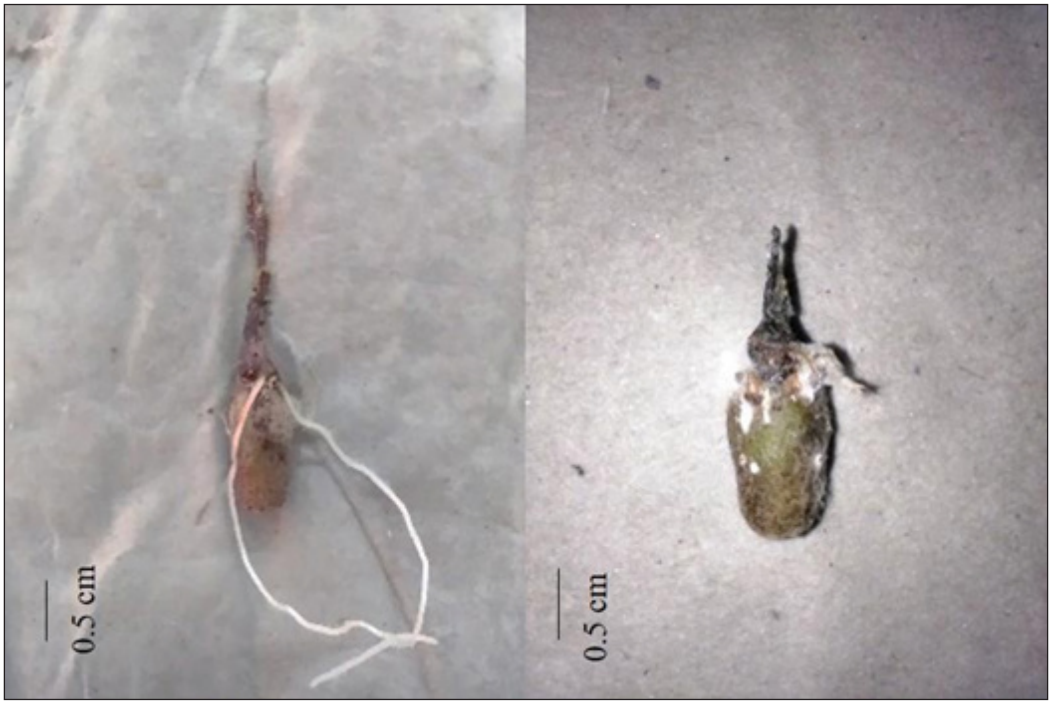

Figure 2. The abnormal seedling caused by primary infection

The optimum storage environment was determined by comparing the moisture content, germination rate, and T50 using ANOVA with a 5 % significance level. When the storage temperature had a significant effect, the analysis continued using DMRT with α=5 %.

RESULTS AND DISCUSSION

Experiment 1: Seed Storage Reserve Composition

Starch was identified as a major component of *A. muelleri* Blume with 51 % content, while protein was 20.76 %, and the lowest content was lipid (1.02 %). Corm of *A. muelleri* Blume is widely used for industrial purposes by extracting the glucomannan content. All part of this plant contains glucomannan, including the seed, which has 15.74 % content (Table 1).

Determination of seed compounds is important due to their role as a reserve source for germination and development. Carbohydrate composition consists of oligosaccharides, including fructans, and polysaccharides such as starch, glucan, and mannan (Aguirre et al., 2018). *A. muelleri* seed not only has starch as a major reserve compound but also glucomannan, which accounts for 15% of the seed reserve. Starch is usually the main polysaccharide stored in seeds, and glucomannan is also a natural macromolecular polysaccharide. The differences in the composition of seed reserves may relate to the germination characteristics. Experiments under stress conditions for Rapeseed cultivars with high oil content showed higher seed germination, vigour, and water uptake than low oil content cultivars (Batool et al., 2022). During seed imbibition, soluble sugars derived from starch are extensively used during the early stages of germination, while soluble proteins are increasingly mobilised as germination progresses. Considering protein contains more hydrophilic radicals than starch, seeds with starch as a significant molecule at the end stage of imbibition had a lower final percentage of water uptake than those in which protein is predominant (Zhao et al., 2018).

Glucomannan has a high viscosity, swelling property, solubility, a good film-forming property, and a good gel property in aqueous solutions (Sun et al*.,* 2023). It can be hydrolysed into monosaccharides using β- mannanase, which catalyses β-D-1,4-mannopyranosyl into mannobiose and mannotriose (Cui et al., 2019).

Table 1
Percentage of A. muelleri *seed reserve components in wet bases*

Seed Reserve Component	%
Starch	51.00
Lipid	1.02
Protein	20.76
Glucomanan	15.74

Experiment 2: Effect of Desiccation On Seed Moisture Content and Viability

Seed desiccation using different rates of drying methods, such as slow (22 °C 10% RH), moderate (35 °C 50% RH), and rapid desiccation (35 ^{O}C 40% RH with warm free airflow) had different effects on moisture content and viability. The duration of desiccation varied from 32 d (moderate drying at 35 °C) to 217 d (slow drying at 22 °C) until it reached a certain moisture content (40-50%; 30-40%; 20-30%). MDN2 and PWTA were dormant since initial germination was lower compared to seed germination after drying treatment.

Seed lots of MDN1, MDN2, and PWTA required 42 days of rapid desiccation method to reduce the seed moisture content from 68.4% to 30.9%, 67.4% to 21.3%, and 67.3% to 43.2 %, respectively. In contrast, seed moisture content only reduced slightly from 68.4% to 55.6%, 67.4% to 62.3%, and 67.3% to 59.8% in 32 days in the moderate desiccation method. Since the seed had germinated, the drying treatment was stopped.

The slow desiccation method required the longest period to decrease the moisture content. The seed moisture content of lots MDN1, MDN2, and PWTA only reduced by 6.2%, 4.8%, and 5.5%, respectively, during 54 days of desiccation. Seed moisture content could not decline to 10-12% using all desiccation methods. Low temperature and RH of slow desiccation inhibited seed germination. The low temperature and RH applied during slow desiccation suppressed seed germination, allowing continued desiccation to identify the lowest moisture content that the seed could tolerate while minimising germinative metabolism. All seed lots reached 38.7-43.8% moisture content after 217 days of slow desiccation treatment (Table 2).

Seed viability before and after desiccation was verified by germination testing. Initial germination of MDN1, MDN2, and PWTA were 73%, 49%, and 79%, respectively. Rapid desiccation treatment using warm free airflow at 35 °C with 40% RH had a lethal effect on the seed. After 42 days of treatment, the seed appeared rotten and mouldy in 10 days of germination testing. Moderate desiccation at 35 °C with 50% RH increased the germination of MDN1, MDN2, and PWTA lots to 87%, 59%, and 89%, respectively, compared to initial germination. Seed responses to slow desiccation differed among seed lots. Germination of MDN2 and PWTA increased to 82.7% and 91.3%, while MDN1 slightly decreased to 78% after 54 days of desiccation treatment. A longer period of rapid desiccation (217 d) reduced seed germination to 48% (MDN1), 59.3% (MDN2), and 68% (PWTA).

The experiment showed that slow desiccation at 22 °C with 10% RH would be suitable for the recalcitrant test for *A. muelleri*. Despite high seed moisture content, low temperature and RH during desiccation prevented seed germination. In contrast, moderate desiccation at 35 °C with 50% RH promoted seed germination after 32 days. These results are consistent with the behaviour of recalcitrant seed, which remains metabolically active and may initiate germinative processes during storage. For example, germination testing of recalcitrant *Trichilia emetica* seed in elevated temperatures up to 6 °C above ambient did

not damage metabolic and ultrastructural integrity in embryonic axes but instead accelerated germinative development and enhanced seedling growth rates (Shersen et al*.,* 2013).

Desiccation treatment affects the germination period. MDN1 and PWTA had shorter germination periods (60 d) after moderate desiccation for 32 d compared to slow desiccation treatment for 52 d (78 d). On the other hand, MDN2 had a shorter germination period (132 d) after 54 d slow desiccation treatment compared to moderate desiccation treatment. A longer period of slow desiccation treatment (217 d) led to a shorter germination period of 45 d (Table 2).

All seed lots preserve the viability (78-91.3%) when the moisture content remains above 50%. Seed germination decreased to 68%, 59.3%, and 48% when the seed moisture content was reduced to 43.8% (PWTA), 42.1% (MDN2), and 38.7% (MDN1), respectively. In addition, viability was lost when the moisture content reduced to 30.9% (MDN1), 21.3% (MDN2), and 43.2% (PWTA) (Table 2).

A different result of experiments was shown, even though both the rapid and moderate desiccation used the same temperature. The use of free airflow in rapid desiccation resulted in a fast loss of seed moisture content and a fall off of seed germination, while moderate desiccation maintained the seed germination. RH had no significant effect on seed viability. Although slow desiccation had the lowest RH, it preserved the seed viability.

A. muelleri seed showed high moisture content at harvest and required a prolonged period to achieve even modest moisture reduction. Seeds are sensitive to desiccation,

Table 2
Seed moisture contents (MC), germination rates and germination periods of A. muelleri *seed after different rates of desiccation*

Dessication Method	Seed Lot	Dessication Period (d)	Initial MC	Final MC	Germination (%)	Germination Period (d)
Slow desiccation at 22 °C 10% RH	MDN 1	54	68.4	62.2	78.0	78
	MDN2		67.4	62.6	82.7	78
	PWTA		67.3	61.8	91.3	78
	MDN 1	217	68.4	38.7	48.0	45
	MDN2		67.4	42.1	59.3	45
	PWTA		67.3	43.8	68.0	45
Moderate desiccation at 35°C 50% RH	MDN 1	32	68.4	55.6	87.0	60
	MDN2		67.4	62.3	59.0	132
	PWTA		67.3	59.8	89.0	60
Rapid desiccation with warm free air flow at 35 °C 40% RH	MDN1	42	68.4	30.9	0	10
	MDN2		67.4	21.3	0	10
	PWTA		67.3	43.2	3.0	10

showing rapid desiccation, but remain viable when the moisture content declines slowly. Viability was preserved when the seed had a minimum water content of 50%. Therefore, *A. muelleri* Blume seed is characterised as a recalcitrant seed.

Similar responses have been reported in previous studies. For example, recalcitrant *Quercus nigra* desiccation at 27 °C for 10 d was suitable, while desiccation at 40 ^{O}C had a detrimental effect (Bonner, 1996). *Embelia tsjeriam-cottam* seed, classified as intermediate and desiccation-sensitive, tolerated moisture content reduction by up to 14%. Sun-drying for three days led to the highest loss of seed germination from 92% to 23.75% during storage at 30 °C after 12 months (Chauhan et al., 2020). In contrast, exposure of recalcitrant seed of *Trichilia emetica* to elevated temperature (5-6 °C above ambient) did not affect germination but enhanced seedling growth rate (Sershen et al., 2013). The desiccation process leads to mechanical, physical membrane, and metabolism-induced damage. Despite high moisture content, seed deterioration occurs due to cell organelles' dysfunction and extensive vacuolization (Lah et al., 2023; Ranganathan & Groot, 2023). Storage of *Ligustrum perrotteiei* seed in the lowest RH (32.4%) and highest rate of desiccation (17.62%) decreased the viability from 100% to 50%, while increasing electrical conductivity from 1.90 dSm^{-1} to 2.67 dSm^{-1} (Aadhavan & Umarani, 2020). Therefore, recalcitrant seeds are sensitive to deterioration either under low or high moisture content.

Experiment 3: Effects of Storage Environment on Seed Germination

Three different environments were applied to determine the optimum temperature and RH for maintaining seed moisture content and viability. Seed moisture content was determined for each month (M0-M5) during seed storage. Initial seed moisture content for L1, L2, L3 were 60.2%, 62.9% and 60.2%, respectively. Each storage environment (S1, S2, and S3) had different effects on moisture content and viability. As the duration of storage increased, there was a rapid decline in moisture content under S1 storage after 3 months, while the decline was more gradual under S2 and S3 conditions (Figure 3).

Storage of S1 showed a significant decrease in seed moisture content to 16.1% (L1), 37.9% (L2), and 24.8% (L3) during 5 months. The moisture content remained above 48%, and germination remained above 80% when stored at S2 (Figure 3). Although seed moisture content remained high (above 46%) under S3 storage, germination sharply decreased after two months, and then viability was lost a month later. The decline in germination and moisture content was in line with S1 storage (Figure 3).

The different storage environments and periods significantly influenced seed germination and T_{50} for all seed lots (Table 3). Initial seed germination of L1, L2, and L3 were 80%, 93%, and 95%, respectively. All seed lots produced a similar pattern of germination and T_{50} result to each different storage environment. Seed germination of S1 storage was steady during three months, then decreased over two months to 50.7% (L1), 30% (L2), and 32% (L3).

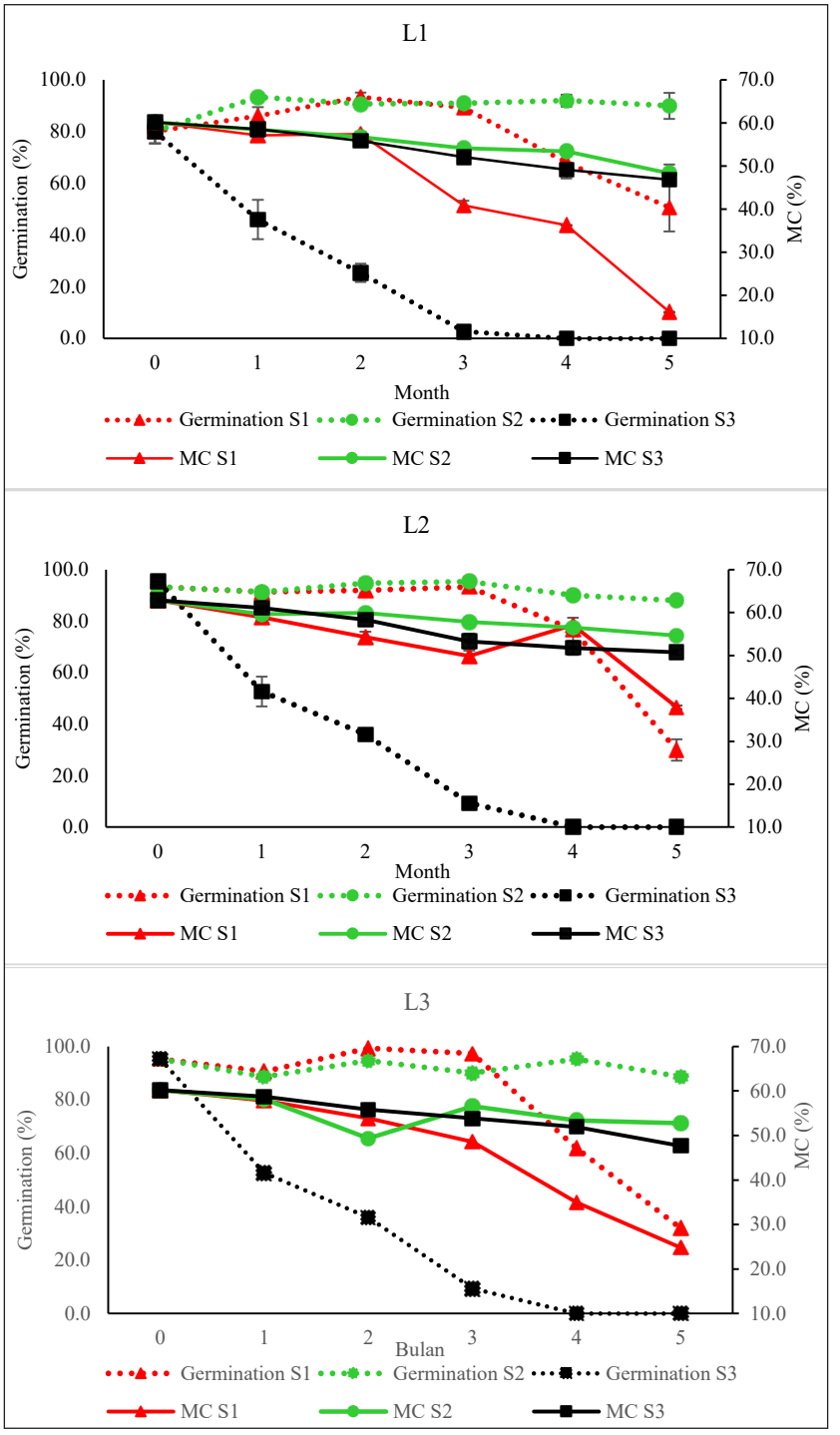

Figure 3. Seed moisture content and germination of lots L1, L2, L3 during five months of storage of S1 (27-32 °C, >60% RH); S2 (20-23 °C, 50-60% RH); S3 (5-7 °C, 40-50% RH)

Meanwhile, germination of S2 storage could preserve seed germination, and there was no significant difference during the 5 months. Germination shortly decreased after a month of storage of S3, then viability was lost after two months of storage (Table 4).

Initial T_{50} showed that L1, L2, and L3 required a long period to obtain a 50% germination rate, namely 92.7 d, 71.4 d, and 86.9 d, respectively. The experiment showed that T_{50} accelerated significantly in the storage of S1 and S2. The lowest T_{50} was reached when L1 (17.0 d), L2 (13.5 d), and L3 (11.8 d) seeds were stored after three months in S1, then increased up to five months. T_{50} reduced steadily during five months of storage in S2 (Table 5).

Table 3
Recapitulation of variance analysis for the effect of seed storage period treatment nested at the storage environment on seed germination and T_{50}

Seed Lot	Source	Germination	T_{50}
L1	S	<0.0001**	<0.0001**
	M(S)	<0.0001**	<0.0001**
	CV	11.9659	12.49667
L2	S	<0.0001**	<0.0001**
	M(S)	<0.0001**	<0.0001**
	CV	6.404195	12.33349
L3	S	<0.0001**	<0.0001**
	M(S)	<0.0001**	<0.0001**
	CV	6.974593	13.80885

Note. S=Storage; M= Storage periods; L= seed lot; CV= coefficient variance; **= values were significantly different according to the anova at $\alpha<0.05$

Table 4
The effect of seed lot and storage environment on seed germination

Seed Lot	Seed Environment	Storage Period (Month)					
		0	1	2	3	4	5
L1	S1 (27-32 °C, >60% RH)	80.0^{ab}	86.0^{ab}	93.3^{a}	89.3^{a}	68.0^{b}	50.7^{c}
	S2 (20-23 °C, 50-60% RH)	80.0^{ab}	93.3^{a}	90.7^{a}	90.7^{ab}	92.0^{a}	90.0^{a}
	S3 (5-7 °C, 40-50% RH)	80.0^{ab}	46.0^{c}	25.3^{d}	2.7^{e}	0.0^{e}	0.0^{e}
L2	S1 (27-32 °C, >60% RH)	93.3^{a}	91.3^{a}	92.0^{a}	93.3^{a}	76.7^{b}	30.0^{d}
	S2 (20-23 °C, 50-60% RH)	93.3^{a}	91.3^{a}	94,7^{a}	95.3^{a}	90.0^{a}	88.0^{a}
	S3 (5-7 °C, 40-50% RH)	93.3^{a}	56.7^{c}	25.3^{d}	0.0^{e}	0.0^{e}	0.0^{e}
L3	S1 (27-32 °C, >60% RH)	95.3^{ab}	90.7^{ab}	99.3^{a}	97.3^{a}	62.0^{c}	32.0^{e}
	S2 (20-23 °C, 50-60% RH)	95.3^{ab}	88.7^{b}	94.7^{ab}	90.0^{ab}	95.3^{ab}	88.7^{b}
	S3 (5-7 °C, 40-50% RH)	95.3^{ab}	52.7^{d}	36.0^{e}	9.3^{f}	0.0^{g}	0.0^{g}

Note. The numbers in the table for each lot, followed by the same letters, show no significant difference at the 5% level DMRT test, respectively

Table 5
The effect of the seed lot and storage environment on T50

Seed Lot	Seed Environment	Storage Period (Month)					
		0	1	2	3	4	5
L1	27-32 °C (>60 % RH)	92.7^{a}	41.4^{e}	20.7^{gh}	17.0^{h}	19.5^{h}	31.6^{f}
	20-23 °C (50-60 % RH)	92.7^{a}	69.1^{c}	41.8^{e}	23.1^{fgh}	22.8^{fgh}	25.6^{fgh}
	5-7 °C (40-50 % RH)	92.7^{a}	53.3^{d}	81.7^{b}	31.0^{gf}	0.0^{i}	0.0^{i}
L2	27-32 °C (±40 % RH)	71.4^{b}	58.2^{c}	17.0^{e}	13.5^{e}	13.8^{e}	37.6^{d}
	20-23 °C (50-60 % RH)	71.4^{b}	71.3^{b}	54.0^{c}	32.8^{d}	51.7^{c}	30.0^{d}
	5-7 °C (40-50 % RH)	71.4^{b}	85.5^{a}	80.2^{ab}	0.0^{f}	0.0^{f}	0.0^{f}
L3	27-32 °C (±40 % RH)	86.9^{a}	32.9^{ef}	14.8^{hi}	11.8^{i}	27.0^{fg}	41.3^{de}
	20-23 °C (50-60 % RH)	86.9^{a}	54.7^{c}	44.5^{d}	30.5^{fg}	27.2^{fg}	22.6^{gh}
	5-7 °C (40-50 % RH)	86.9^{a}	80.0^{a}	66.6^{b}	31.0^{fg}	0.0^{j}	0^{j}

Note. The numbers in the table for each lot, followed by the same letters, show no significant difference at the 5% level DMRT test, respectively

The reduction is consistent with declining moisture content, showing in ambient and controlled storage. This is because embryo development occurred during storage, resulting in faster germination. As a recalcitrant species, *A. muelleri* seeds retain high moisture content at harvest, allowing metabolic activity to continue during storage and contributing to changes in germination behaviour over time.

Different environments of storage affected seed physical appearance. Ambient temperature (S1) allowed seed active metabolism, which resulted in germination. The unavailability of water prevented seedling development. The seed appeared wrinkled and lost the ability to produce normal seedlings. Although the seed in S3 appeared firm, viability was lost after three months due to unbearably low temperatures. Low temperature and high moisture content might lead to seed chilling injury. Storage of S2 was able to suppress seed germination and maintain viability for five months (Figure 4).

This experiment proved that the storage environment is closely related to seed viability. The higher temperature of S1 sped up germination and T50, but only lasted three months. Seed germination started to decline, and T50 increased in the fourth month of storage in S1. Storage of S2 was identified as the most optimum to maintain viability since seed germination was steady and T50 declined in five months. Seed stored under S3 conditions could not maintain viability, showing that low-temperature storage is unsuitable for this species.

Based on the results, temperature affects seed viability and longevity during storage. Recalcitrant seed with high moisture content had an active metabolism of cells. This implies that seed storage at higher temperatures increases several metabolic processes, causing faster deterioration. On the other hand, low storage temperatures with high moisture content also caused a lethal effect. Seed did not tolerate temperatures below 7 °C, while controlled

Figure 4. Seed appearance after a month (A) and five months (B) storage of S1 (27-32°C, >60% RH); S2 (20-23 °C, 50-60% RH); S3 (5-7 °C, 40-50% RH)

storage at 20-23 °C provided the optimum storage condition since it could decrease seed moisture content slightly and preserve viability.

A. muelleri Blume can be stored either up to five months under controlled storage (20-23 °C) or three months under ambient storage (≥27 °C). Other recalcitrant seed such as *Morinda citrifolia,* had short-term storage (2-6 weeks) in temperature of 20-25 °C (Ajongbolo et al. 2025). The germination of *Anonna sylvatica* seed decreased during three-month storage in a cold chamber (16 °C and 40 % RH) (Silva et al. 2024). Seeds with high moisture content (≥ 60 %) need oxygen to maintain metabolism. Therefore, a paper bag container is suitable for seed storage, since it allows gas exchange and a good water vapour transmission rate.

An optimum environment for seed storage is required to maintain quality. Temperature is a crucial factor in preventing seed deterioration. Viability is not only affected by storage temperature but also by seed moisture content. Seed age during storage leads to a decrease in viability when there are no suitable conditions. The metabolic system starts to break down when the seed deteriorates, resulting in slow or failed germination and poor seedling development. Therefore, optimum seed storage slows down the seed metabolism without incurring damage.

Temperature and moisture content are the main factors that affect seed deterioration and viability loss. The viability of recalcitrant seed can be maintained when moisture content decreases slightly below full imbibition but remains above levels that induce chilling injury (Vitis et al., 2020). High humidity and temperature during storage are conducive to the immediate germination of recalcitrant seed. Therefore, optimum temperature and humidity are needed during seed storage to suppress the rate of seed metabolism and respiration, thereby delaying seed germination.

CONCLUSION

In conclusion, *A. muelleri* Blume was found to have high starch seed (51%) with 15.74% glucomannan content. The viability of seed can be maintained at 40-50% moisture content using a slow-degradation method (silica gel at 22 °C and 10% RH). Seed with a moisture content of around 60% may be stored at controlled temperatures (20-23 °C, 50-60% RH) for five months and for three months at embient temperature (26-32 °C, RH >60%). However, the seed cannot withstand storage at low temperatures below 7 °C. These characteristics confirm that the seeds are recalcitrant.

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Synergistic Application of Marine-derived Actinomycetes and Microalgae to Enhance Rice Seedling Growth and Salinity Stress Tolerance

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ABSTRACT

Rice productivity on marginal land is significantly affected by salinity stress. However, biological approaches to improve rice growth tolerance remain insufficiently explored. This study aimed to evaluate the potential use of various actinomycete strains (SD 008, SD 021, SD 027), microalgae (*Chlorella* sp.), and their consortia to improve rice seedling growth and the physiological responses under different salinity levels (0, 50, and 100 mM NaCl). Rice seedling growth was assessed using several parameters, including seedling height, root length, number of roots, number of leaves, and biomass. In addition, the physiological condition of the plants was analysed using stress indicators consisting of chlorophyll *a* and *b* contents, proline, and malondialdehyde (MDA) accumulation. The results showed that treatment with strain SD 027 and the actinomycetes-microalgae consortium had a significant effect on several growth parameters. This treatment was able to increase the germination index, maintain root development, and enhance chlorophyll content under moderate to high salinity conditions. Furthermore, the increase in proline content, coupled with a decreased MDA level, indicates an improvement in plant tolerance to oxidative stress. Our findings indicate that the combination of actinomycetes and microalgae provides a synergistic effect in enhancing rice seedling growth under salinity conditions. This approach has the potential to be developed as a more

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environmentally friendly strategy to support plant growth on degraded land. However, further field trials are required to evaluate its effectiveness before it can be implemented in broader agricultural practices.

Keywords: Actinomycetes, MDA, microalgae, paddy seedling, proline, saline stress

INTRODUCTION

Salinity is one of the major abiotic stresses that significantly impairs crop productivity, particularly in rice (*Oryza sativa* L.) According to a 2021 report by the World Food Programme, millions of people in various countries still face severe levels of hunger (The World Food Programme, 2021). According to data from the Indonesian CBS in 2020, land conversion in Indonesia reaches 100,000 hectares annually, while the government's ability to open new rice fields is only around 40,000 hectares per year. Of the 189.1 million hectares of total land area in Indonesia, 157.2 million hectares consist of acidic soils, with the majority classified as dry acidic soils, accounting for approximately 108.8 million hectares or more than 60% of the total dry land in Indonesia. These types of soils are spread across almost all regions of Indonesia, from Kalimantan, Sumatra, parts of Sulawesi, to Papua (Centre Bureau of Statistics, 2020).

Suboptimal soils are characterised by reduced availability of essential macronutrients and elevated concentrations of toxic ions. The main issue with suboptimal land types is the reduction in nutrient solubility, particularly soluble phosphorus (P). Additionally, there is a decrease in the concentration of macroelements such as nitrogen (N), magnesium (Mg), calcium (Ca), and potassium (K), along with an increase in non-essential elements that can lead to plant toxicity (AbdelRahman et al., 2022). The efforts to improve the fertility of submarginal soils increasingly involve the use of microorganisms, which have become an important focus in sustainable agriculture research (Sadak & Dawood, 2023). Nitrogen-fixing bacteria, such as *Rhizobium* and *Azospirillum,* have long been reported to play a role in increasing nitrogen availability in submarginal soils. Several recent studies have also highlighted that these bacteria play an important role in supporting improved plant productivity in soils with low nitrogen content (Silva et al., 2022). The symbiosis between mycorrhizal fungi and plants has been recognised as contributing to the improvement of phosphorus availability and other essential nutrients in submarginal soils (Sadak, 2023). In addition, various studies have reported that actinomycetes and microalgae play an important role in enhancing plant growth under abiotic stress conditions. This role may occur through several biological mechanisms, including phytohormone production, nutrient Solubilisation, and modulation of the plant antioxidant system.

Several previous studies have indicated that the genus *Streptomyces* plays a role in enhancing plant tolerance to environmental stresses. Furthermore, microalgae have also

been reported to increase salt tolerance in tomato plants (*Solanum lycopersicum*) (Chen et al., 2025). Microalgae such as *Chlorella vulgaris* are also known to stimulate root elongation and shoot development in *Arabidopsis thaliana* under salinity stress by regulating proline accumulation and reactive oxygen species (ROS) levels (Fiorentino et al., 2025).

Various efforts have been undertaken to increase crop productivity through the application of modern agricultural technologies, including drainage systems, soil tillage, and the use of crop varieties that are tolerant to environmental stresses. Although these approaches provide certain benefits, their implementation often presents several challenges. One of the main limitations is the high financial investment required, which means that such technologies are not always accessible to smallholder farmers, particularly in developing countries (Midgley et al., 2017).

In addition, practices such as drainage and soil tillage may generate negative environmental impacts. Land degradation and disruption of natural ecosystems are among the long-term environmental impacts that may arise from these practices (Dai et al., 2022). Dependence on these technologies may also make farmers more vulnerable when these technologies become unavailable due to limited purchasing power or other supply constraints.

Microorganism-based approaches involving soil microbes have been widely reported as a key sustainable strategy to enhance environmentally friendly crop productivity. Actinomycetes and microalgae are groups of microorganisms with considerable potential for such applications. Actinomycetes are known for their ability to solubilise phosphate, fix atmospheric nitrogen, and produce various plant growth-promoting compounds such as indole-3-acetic acid (IAA). On the other hand, microalgae such as *Chlorella* sp. play an important role in increasing soil organic matter content, improving soil structure, and enhancing plant photosynthetic efficiency (Djebaili et al., 2020). The consortium of these two microorganisms is considered complementary, contributing to improved nutrient availability for plants (Bakhoum et al., 2023; Llamas et al., 2023).

The use of microbial consortia represents a highly promising approach in the development of more environmentally friendly and sustainable agricultural systems. This is closely related to the ability of microorganisms to reduce dependence on synthetic chemical fertilisers (Olanrewaju & Babalola, 2019). However, studies examining the interaction between locally derived tropical actinomycetes and microalgae as potential agents in the formulation of biostimulant technologies for improving submarginal soils remain very limited. This gap in scientific knowledge highlights the need for further exploration of the synergistic effects between these two groups of microorganisms, particularly regarding their role in improving soil health and enhancing crop productivity in tropical submarginal agricultural systems (Kusvuran, 2021).

The role of microorganisms in enhancing plant tolerance to various environmental stresses has been widely reported in previous studies. However, the interaction between actinomycetes and microalgae derived from tropical marine ecosystems has received relatively limited attention. Therefore, this study was designed to explore the potential of these microorganisms in improving the tolerance of rice seedlings to salinity stress during the early growth stage. Specifically, this research evaluates the effectiveness of actinomycetes isolates, both individually and in consortium with microalgae, in mitigating the negative effects of NaCl-induced salinity. The findings of this study are expected to contribute to the development of more sustainable biological approaches for enhancing rice resilience in environments with high salinity levels.

MATERIALS AND METHODS

Cultivation of Actinobacteria and Microalgae Strains

Selected actinomycete strains were rejuvenated on Starch Nitrate Agar (SNA) medium (prepared using seawater per litre of medium) for 7 days until spore formation occurred. Subsequently, each strain was cultured in starch nitrate broth (SN broth) by transferring an agar block (approximately 1.5 cm^2) containing colonies from the SNA medium. The cultures were incubated on a shaker at an agitation speed of 150 rpm at 30 °C for 7 days. The culture broth was then transferred into ISP2 (International Streptomyces Project) fermentation medium at 10% (v/v) with a cell density of approximately 10^7 cfu/mL for biomass production. The obtained biomass was washed three times with sterile distilled water to separate the biomass from the culture supernatant. The effects of actinomycetes and microalgae treatments on rice seed germination were evaluated through seed soaking under salinity stress conditions induced by NaCl at concentrations of 0, 50, and 100 mM. Seed germination responses were subsequently assessed at each level of salinity treatment.

In this study, microalgae of the genus *Chlorella* sp. were recovered from marine sponges and subsequently cultured in 300 mL of Walne medium contained in 1.5 L flasks under controlled laboratory conditions. The cultures were continuously aerated to ensure sufficient oxygen supply and proper mixing. A constant lighting system was maintained to provide continuous illumination for 24 hours throughout the experimental period. The cultures were incubated at room temperature for 7 days to allow the microalgae to grow and accumulate biomass optimally under these conditions.

Evaluation of Plant Growth-promoting Potential

To assess the potential for plant growth-promoting properties, each isolate was tested for its ability to produce indole-3-acetic acid (IAA), solubilise phosphate, and nitrogen fixation. These tests were conducted to identify the functional characteristics of the isolates that play a role in supporting plant growth.

Indole-3-Acetic Acid (IAA) Production Activity

Indole-3-acetic acid (IAA) production in selected isolates was analysed to determine their potential plant growth-promoting activity. Each isolate was inoculated into ISP2 broth enriched with 0.5% tryptophan as a precursor for IAA biosynthesis. The cultures were then incubated at 30°C for 48-72 hours with shaking at 150 rpm to support microbial growth and metabolite production.

After the incubation period, the cultures were centrifuged at 10,000 rpm for 10 minutes to separate the bacterial cells from the culture supernatant. The supernatant was then used to determine IAA concentration using a colourimetric method with Salkowski's reagent, as described by Guardado-Fierros et al. (2024). For this purpose, 1 mL of the supernatant was mixed with 2 mL of Salkowski reagent, which consists of 2% ferric chloride ($FeCl_3$) in 35% perchloric acid. The mixture was then incubated in the dark at room temperature for 20-30 minutes until a pink colour formed, indicating IAA production. The intensity of the resulting colour was then measured using a spectrophotometer at a wavelength of 530 nm.

Phosphate Solubilisation Activity

Selected representative isolates were inoculated into the centre of Pikovskaya agar, a medium containing an insoluble phosphate source in the form of calcium phosphate ($Ca_3(PO_4)_2$). The culture plates were then incubated at 37°C for 7 days to allow sufficient time for microbial growth and the phosphate solubilisation process.

Phosphate solubilisation ability was observed through the formation of a clear zone (halo) around the microbial colony, indicating that the insoluble phosphate had been converted to a more soluble form. To increase data reliability, each test was performed in duplicate.

Furthermore, the solubilisation index (SI) was calculated quantitatively by comparing the total diameter, including the colony and the halo zone, to the colony diameter. This index calculation followed the method reported by Chakdar et al. (2018) using the following formula:

$$SI = \frac{\text{Total diameter (Colony + Halo zone)}}{\text{Colony diameter}}$$

Nitrogen Fixation Activity

The nitrogen fixation ability of selected isolates was evaluated using Burk's nitrogen-free medium with the following composition (g/L of distilled water): 10% glucose, 0.41% KH_2PO_4, 0.05% Na_2SO_4, 0.52% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% $CaCl_2$, 0.0025% $Na_2MoO_4 \cdot 2H_2O$, 0.005% $FeSO_4 \cdot 7H_2O$, and 15% agar, with the pH maintained at 7 ± 0.1. This medium is used to assess the ability of microorganisms to fix atmospheric nitrogen

because it does not contain an external nitrogen source. Evaluation was performed by inoculating isolates into Burk's nitrogen-free medium in either agar or broth form. For qualitative observation, isolates were streaked onto Burk N-free agar and then incubated at 30 °C for 5-7 days.

The ability of an isolate to grow in nitrogen-depleted media indicates potential nitrogen fixation activity. Furthermore, a blue colour change in the medium surrounding the colony serves as an additional indicator of the isolate's ability to fix atmospheric nitrogen.

Seed Viability Test

Rice (*Oryza sativa* L.) seeds were surface sterilised using 70% ethanol for 1 minute, then immersed in a 2% sodium hypochlorite solution for 10 minutes. Finally, the seeds were rinsed with sterile distilled water to remove any remaining sterilant. Seed viability was assessed using a germination test. Seeds were sown in Petri dishes containing moistened filter paper and incubated at room temperature for 7 days. Germination percentage was recorded to determine seed viability across different treatments. Germination was defined as the emergence of the radicle and endosperm. The germination of rice seeds was monitored and recorded daily to determine germination potential (%), germination rate (%), and the germination index. At the conclusion of the germination test, the radicle length, radicle thickness, and the length of young rice shoots were measured using a straightedge (Ullah et al., 2024).

Germination potential (%) was calculated using Equation 1:

$$\text{GP} = \frac{G2d}{Gt} \times 100 \quad [1]$$

where *G2d* is the amount of germinated seeds in the first two days; *Gt* is the total amount of test seeds.

Germination rate (%) was calculated using Equation 2:

$$\text{GE} = \frac{Gf}{Gt} \times 100 \quad [2]$$

where *Gf* is the amount of germinated seeds when the seed reaches full germination; *Gt* is the total amount of test seeds.

Germination Index was calculated using Equation 3:

$$\text{GI} = \sum(Gt/Dt) \quad [3]$$

where *Gt* is the amount of germination on day t; *Dt* is the corresponding amount of days to germination.

Growth Experiment

The experiment was conducted using rice seedlings grown under controlled laboratory conditions. Seeds were initially pre-germinated for 3 days in distilled water before being transferred to growth media containing NaCl concentrations of 0 mM (control), 50 mM, and 100 mM. Each treatment consisted of five groups: (1) control (no biological treatment), (2) strain SD 027, (3) strain SD 021, (4) strain SD 008, (5) microalgae, and (6) a consortium of actinomycetes with microalgae. Seedlings were grown for 14 days, and growth parameters, including seedling height, root length, root number, leaf number, and biomass, were recorded at the end of the experiment.

Chlorophyll Content of Paddy Seedlings

Chlorophyll *a* and *b* were extracted from fresh leaves using 80% acetone. Approximately 0.1 g sample of fresh leaves was first ground with acetone, then centrifuged at 10,000 rpm for 10 minutes to separate the chlorophyll extract from the residual leaf tissue. The resulting supernatant was then collected and analysed using a spectrophotometer, measuring absorbance at wavelengths of 663 nm for chlorophyll *a* and 645 nm for chlorophyll *b*.

Furthermore, chlorophyll content was calculated using the method proposed by Pérez-Patricio et al. (2018), and the results were expressed in mg/g fresh leaf weight.

$$\text{Chlorophyll } a \text{ (mg/g FW)} = (12.7 * A_{663}) - (2.59 * A_{645})$$

$$\text{Chlorophyll } b \text{ (mg/g FW)} = (22.9 * A_{645}) - (4.7 * A_{663})$$

The absorbance values, A663 and A645, were recorded at wavelengths of 663 nm and 645 nm, respectively. The spectrophotometer was calibrated using an acetone/ethanol mixture.

Proline Accumulation Assay

The proline content of roots and leaves of paddy seedlings was measured following the procedure described by Gharsallah et al. (2016). Approximately 0.5 g of sample was ground and homogenised in 3% sulfosalicylic acid, then centrifuged at 3000 rpm for 10 minutes to separate the supernatant from the remaining tissue. The supernatant was mixed with acid-ninhydrin reagent and glacial acetic acid, then incubated at 100°C for 1 hour. The mixture was then extracted with toluene to extract the organic phase containing proline.

The absorbance of the toluene phase was measured at a wavelength of 520 nm using a spectrophotometer. Proline content was determined based on a standard curve, and the results were expressed as μmol/g fresh weight.

Malondialdehyde (MDA) Analysis

Lipid peroxidation was assessed by measuring MDA content in roots and leaves as an indicator of oxidative stress (Patanè et al., 2022). Samples (0.5 g) were homogenised in 0.1% trichloroacetic acid (TCA) and centrifuged at 12,000 rpm for 15 minutes. The supernatant was mixed with 0.5% thiobarbituric acid (TBA) in 20% TCA and heated at 95°C for 30 minutes. After cooling, the mixture was centrifuged, and the absorbance was measured at 532 nm, 600 nm and 450 nm. MDA concentration was calculated using the extinction coefficient and expressed in nmol/g fresh weight.

$$\text{MDA (nmol/g FW)} = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$$

Morphological and Physiological Characterisation

The morphological and physiological characteristics of *actinomycetes* strains were determined using M1 growth media. Strains SD 008, SD 021, and SD 027 were morphologically characterised as Gram-positive filamentous bacteria and further identified through 16S rRNA gene sequencing. The gene was amplified using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3').

The DNA sequences were edited and analysed using BioEdit, then aligned to reference sequences using ClustalW in MEGA11. Similarities to known organisms were checked using BLASTn in the NCBI GenBank database. A phylogenetic tree was then constructed using the neighbour-joining method, and branch reliability was validated using 1,000 bootstrap replicates.

The entire experimental workflow is depicted in a flowchart. This chart summarises the step-by-step evaluation of the effect of actinomycete and microalgae treatments on rice seedling growth under various levels of NaCl-induced salinity stress. The diagram also explains the inoculation process, salinity treatment, incubation period, and physiological and biochemical parameters measured at 14 days post-treatment (DAP) (Figure 1).

Data Analysis

Each experiment was conducted in triplicate to ensure the reliability of the results. The data obtained were analysed using one-way ANOVA, followed by a post-hoc Tukey test to evaluate significant differences between treatments. Results were considered statistically significant if $p < 0.05$.

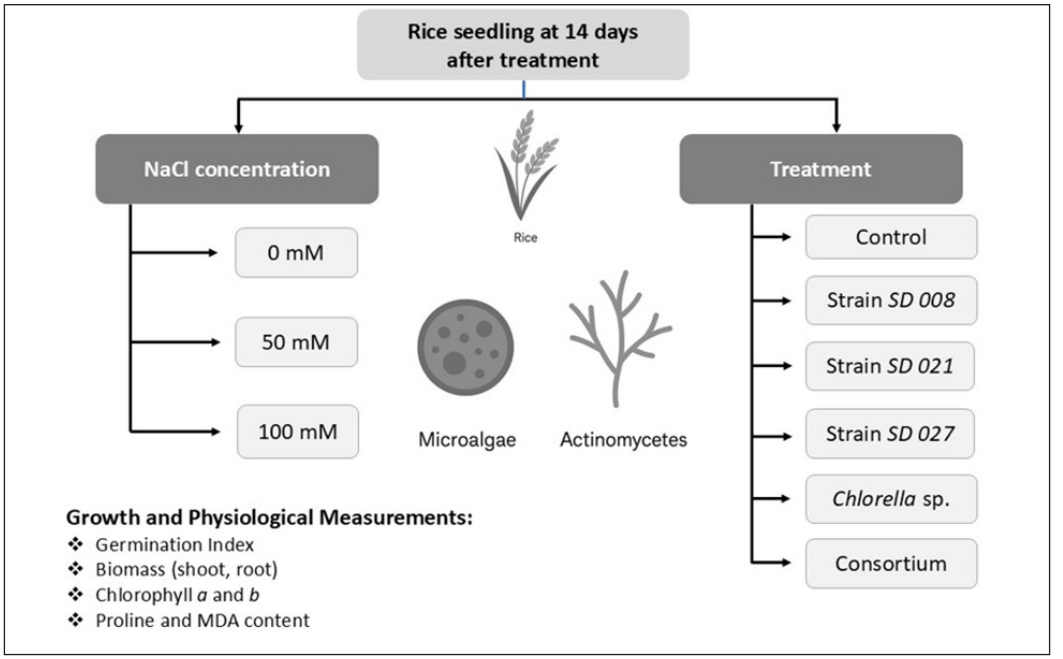

Figure 1. Flowchart of the experimental procedure for evaluating rice plant salt tolerance, microbial-induced

RESULTS AND DISCUSSION

Characteristic of Strains

Actinomycetes that were obtained from marine sponges exhibited morphological and physiological diversity when cultured in the laboratory. Substrate mycelium was observed in two distinct colours, brown and grey, while aerial mycelium showed a variety of colours ranging from yellowish brown to ivory white to pure white (Figure 2). These pigmentation patterns are consistent with the characteristics of the genus *Streptomyces*, which is known to produce a variety of pigments as secondary metabolites.

Optimal growth of the isolates was observed at 35-37 °C, indicating their adaptation to mesophilic conditions. Furthermore, the isolates also grew well at a neutral pH (pH 7), consistent with the pH conditions of their native marine environment (Table 1).

Regarding salinity tolerance, the isolates were able to grow in media containing 5-7% NaCl, confirming their halotolerant nature. This highlights the ability of marine actinomycetes to survive and thrive in habitats with moderate salt concentrations, a characteristic common among marine microbes.

Actinomycetes obtained from marine sponges exhibit unique morphological and physiological characteristics, most notably their spirally arranged spore chains.

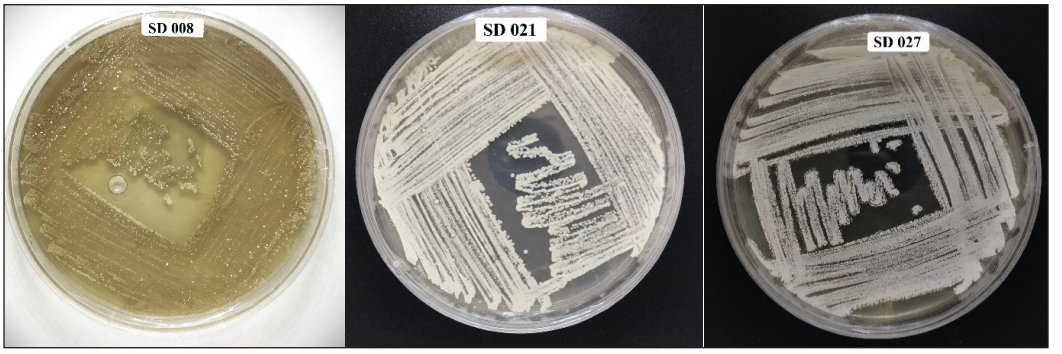

Figure 2. Morphological characteristics of the strain colony were grown on M1 medium (pH 7±0.2) at 30°C for 10 days

Table 1
The morphological and physiological traits of selective actinomycetes strains grown on different media

Parameter	Strain SD 008	Strain SD 021	Strain SD 027
Morphological characteristics			
Colony colour	Light brown	Ivory	White
Texture	Smooth	Powdery	Velvet
Aerial mycelium	Yellowish-brown	Ivory-white	White
Substrate mycelium	Brown	Brown	Grey
Spore chain ornament	Open coil spiral	Tight coil spiral	Open coil spiral
Pigmentation	Brown	-	-
Physiological characteristics			
Optimal growth temperature (°C)	35	35	37
Optimal pH for growth	7	7	8
Salt tolerance (%)	5	5	6

Note. All strains were grown on M1 medium (pH 7±0.2) at 30°C for 10 days

This characteristic is typically associated with the genus Streptomyces, known for its filamentous structure and spore-forming ability. The spiral arrangement of the spore chains facilitates the dispersal and survival of actinomycetes in marine environments, where nutrient availability and ecological pressures frequently fluctuate (Jackson et al., 2018; Riahi et al., 2019). Similar observations have been found in other marine actinomycetes, suggesting that this morphological adaptation provides advantages in colonisation and resilience in the complex sponge microhabitat (Jagannathan et al., 2021).

The dominant colony colours observed in actinomycetes isolated from marine environments, including sponges, are reflective of their adaptation to the oligotrophic and high-salinity conditions of the sea. In this study, most actinomycete colonies were white, grey, and pale yellow.

These colours are characteristic of *Streptomyces* species and have been widely documented in marine isolates (Naligama et al., 2022). Recent research has shown that actinomycetes associated with marine sponges often exhibit higher metabolic diversity compared to their terrestrial counterparts, as evidenced by their genomic and biosynthetic capabilities (Xu et al., 2023). Overall, the observed traits in actinomycetes, including spiral spore chains and dominant colony colours, are aligned with findings reported in recent marine microbiology studies.

Phylogenetic Tree Analysis of Strains

The 16S rDNA gene sequences of strains isolated from marine sponges were analysed to determine their phylogenetic relationships. BLAST analysis against the NCBI database revealed that all strains showed high sequence similarity to known species in the genus *Streptomyces* (Figure 3). The resulting phylogenetic tree indicated that the strains were closely related to Streptomyces species previously reported from marine environments. The nucleotide sequence differences observed among the isolates, despite their similarity to strains in the database, suggest that these strains may represent unique variants within the genus Streptomyces.

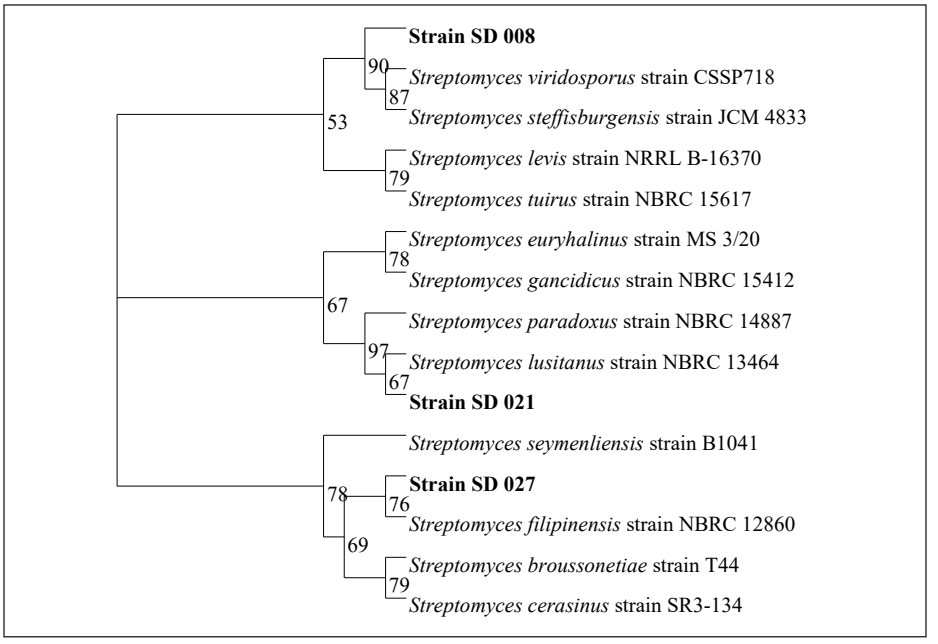

Figure 3. The phylogenetic relationships of strains isolated from marine sponges with related genera were inferred using the Neighbour-Joining method based on 16S rDNA gene sequences. Bootstrap values, calculated from 1000 replicates, are displayed as percentages at the branch points. The scale bar indicates 1 nucleotide substitution per 100 nucleotides

The phylogenetic analysis of strains isolated from marine sponges based on their 16S rDNA sequences revealed a close relationship to members of the genus *Streptomyces*. This genus is well-known for its remarkable diversity and its capacity to produce secondary metabolites with various biological activities, including antimicrobial and anticancer properties (Sarkar & Suthindhiran, 2022). The high bootstrap values observed in the phylogenetic tree confirmed the robustness of the clustering and the genetic relatedness of the strains to known *Streptomyces* species in the NCBI database. Previous studies have reported that *Streptomyces* genera often exhibit unique biosynthetic pathways and metabolic profiles, particularly when isolated from marine environments (Almeida et al., 2019). Marine *Streptomyces* strains are known for their remarkable adaptation to extreme marine conditions (Tangerina et al., 2020). The phylogenetic relationship of this isolate to other *Streptomyces* species is also consistent with findings reported in a similar study of actinomycetes from marine sponges (Chen et al., 2021).

Biological Activity of Strains

The ability of three actinomycete strains (SD 008, SD 021, and SD 027) to solubilise phosphate and produce indole-3-acetic acid (IAA) was evaluated to assess their potential as plant growth promoters. Phosphate solubilisation index (PSI) and IAA production values differed among the strains (Table 2). These differences in PSI values reflect the varying ability of each strain to release soluble phosphate from insoluble sources. The mechanism of phosphate solubilisation is primarily through the production of organic acids, which lower the pH of the surrounding medium and facilitate phosphate release (Boubekri et al., 2021). Strain SD 021, with the highest PSI (2.71), shows promising prospects as a phosphate-solubilising microorganism, indicating its strong capacity to increase phosphorus availability in the rhizosphere.

IAA production is an important plant growth-promoting trait, regulating root elongation, lateral root formation, and overall plant development. All three actinomycete strains exhibited significant IAA production, with strain SD 021 having shown the highest levels (116.21 µg/mL). The ability of these strains to synthesise large amounts of IAA highlights

Table 2
Physiological activity of selected actinomycete strains grown on M1 media

Strain	Phosphate Solubilisation [SI]	N_2-fixation	IAA Production (µg mL-1 ± SD)
SD 008	+ (1.52)	+	99.25±7.43
SD 021	+ + (2.71)	+	116.21±6.44
SD 027	+ + (2.11)	+	102.43±2.47

Note. The symbol + represents a positive reaction, - represents a negative reaction

their potential role in enhancing plant growth by promoting root system development and nutrient uptake (El-Bassiouny et al., 2020; Li et al., 2021).

The combination of phosphate solubilisation and IAA production in these strains represents a characteristic of marine Streptomyces species, which are often associated with enhanced plant growth. Previous studies have reported that *Streptomyces* spp. Isolated from marine environments, it not only tolerates extreme conditions but also contributes to plant growth under stress, such as saline soils or nutrient-deficient environments (Boukhatem et al., 2022; Romano-Armada et al., 2020).

Of the three actinomycetes strains, SD 021 showed higher biological activity in the process of phosphate solubilisation and IAA production. This suggests that SD 021 could be further explored as a biofertilizer candidate, particularly for improving crop productivity in nutrient-limited soils. However, additional *in planta* experiments are necessary to confirm these beneficial effects under field conditions.

Effect of Actinomycetes and Microalgae Treatments on Rice Seedlings Under NaCl Stress

Germination results under varying NaCl concentrations (0 mM, 50 mM, and 100 mM) revealed that the distilled water (control) treatment was consistently achieving the highest germination percentage (GP), average germination percentage (GE), and germination index (GI) across all conditions (Table 3) (Figure 4). Under the 0 mM NaCl treatment, control seeds were recorded to have reached GP, GE, and GI values of 95%, 95%, and 3.16, respectively. Moderate salinity (50 mM) was found to slightly reduce these values to 90%, 95%, and 3.16, while high salinity (100 mM) further decreased them to 85%, 100%, and 3.33.

Table 3
Response of rice seedlings under NaCl stress treated with actinomycetes and microalgae

Treatment	Seedling Percentage								
	0 mM NaCl			50 mM NaCl			100 mM NaCl		
	GP	GE	GI	GP	GE	GI	GP	GE	GI
Aquadest	95	95	3.16	90	95	3.16	85	70	3.33
SD 008	70	90	3	45	100	3.33	65	100	3.33
SD 021	70	80	2.6	40	95	3.16	20	65	2.16
SD 027	25	100	3.33	80	100	3.33	30	45	3
Chlorella sp.	65	90	3.33	60	85	2.8	35	80	2.67
SD+ *Chlorella* sp.	80	95	3	85	95	3.16	80	100	3.33

*GP, germination potential; GE, germination rate; GI, germination index

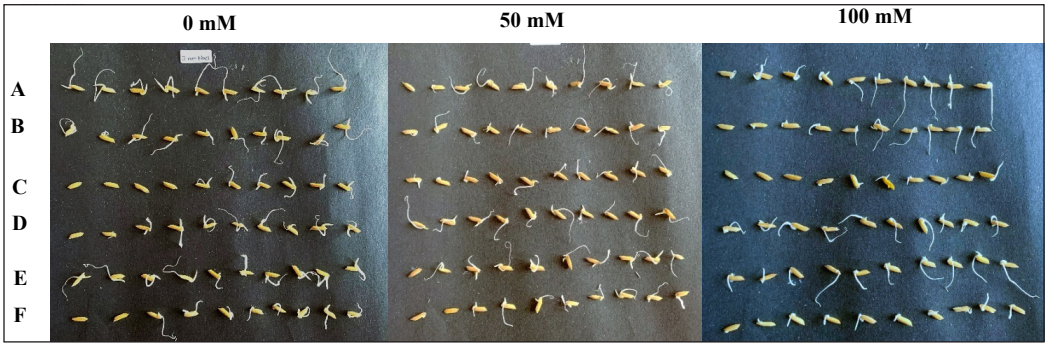

Figure 4. Growth profile of 14-day-old rice seedlings cultivated under NaCl stress conditions and treated with actinomycetes and microalgae

Note. (A) Aquadest/water; (B) SD 008; (C) SD 021; (D) SD 027; (E) *Chlorella* sp.; (F) consortium of actinomycetes strains and microalgae SD+*Chlorella* sp.

The combination of SD strains and microalgae was consistently observed to outperform individual treatments under stress conditions, resulting in GP, GE, and GI values of 80%, 100%, and 3.33 at 100 mM NaCl. This study highlights the potential of combining microbes and microalgae to promote seed germination under salinity stress.

Treatment with various actinomycetes was observed to show a positive effect on the germination ability of rice seeds under NaCl-induced stress at concentrations of 0, 50, and 100 mM (Figure 5). The results of this study indicate that microbial treatments, specifically strain SD 027 and the actinomycete-microalgae consortium, significantly improved rice seedling performance under saline conditions. At NaCl concentrations of 50 mM and 100 mM, the consortium was able to increase the germination index (GI) and maintain better root length and number compared to the control without treatment. These effects indicate a synergistic interaction between microalgae and actinomycetes. These effects are shown in increased photosynthesis and antioxidant production, while actinomycetes increase nutrient availability and produce phytohormones such as IAA. These findings are consistent with previous studies, which have shown that actinomycetes can enhance plant growth and stress tolerance by increasing nutrient availability, producing phytohormones, and inducing systemic resistance (Santoyo et al., 2021).

These findings are consistent with previous studies showing that actinomycetes and microalgae have been able to enhance seed germination by increasing nutrient availability and stress tolerance under favourable conditions (Msimbira et al., 2020). The beneficial effects of microbial consortia in mitigating salt stress, through mechanisms such as osmotic regulation and biofilm formation, have been widely reported (Xiong et al., 2019; Yujia et al., 2023). Our findings highlight the synergistic effect of combining actinomycetes and

microalgae, which was shown to enhance germination even under severe salinity stress, consistent with observations by Carillo et al. (2020). Although treatments with either actinomycetes or microalgae alone were able to produce positive effects, the combined application of both microorganisms significantly improved germination across a range of salinity levels. This enhanced performance is likely due to the microbes' ability to produce growth-promoting compounds, reduce oxidative stress, and maintain ion balance. Overall, these results support the reported evidence regarding the practical value of microbial consortia in managing agricultural systems under salinity stress (Ahluwalia et al., 2021).

Among microbial treatments, SD 008 and microalgae were demonstrated to have the potential to sustain germination under salt stress. At 100 mM NaCl, SD 008 was observed to maintain GE (100%) and GI (3.33) despite a lower GP (65%). Similarly, microalgae were found to achieve GE of 80% and GI of 2.67, but with a reduced GP (35%). Treatments with SD 027 and SD 021 were shown to exhibit diminished performance under increasing salinity, particularly at 100 mM, where GP dropped to 30% and 20%, respectively.

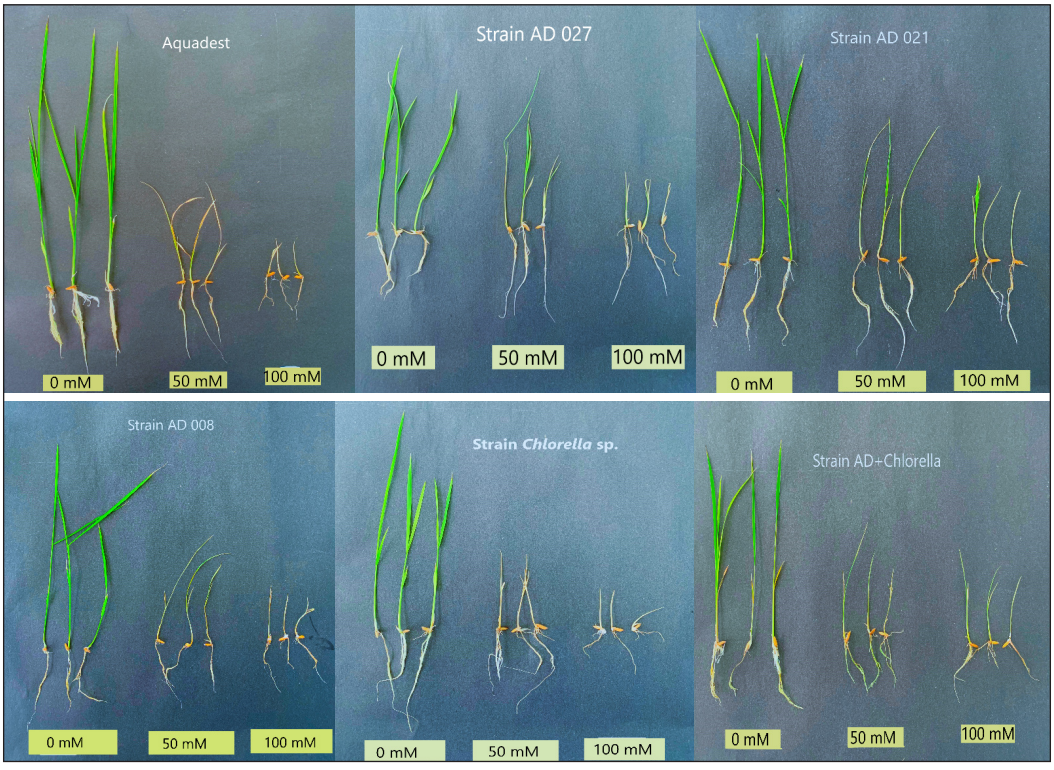

Figure 5. In vitro growth of rice seedlings under salinity levels (0, 50, and 100 mM NaCl) with actinomycetes and microalgae treatments at 14-day-old rice seedlings

Growth of Rice Seedlings In Vitro

Based on in vitro evaluation of rice seedlings after 14 days seedling height, root length, number of leaves, root count, and biomass under treatments with microbes and microalgae were measured at 0 mM, 50 mM, and 100 mM NaCl (Figure 4 and Figure 6) (Table 4) Under 0 mM NaCl, distilled water (control) produced the tallest seedlings (24.79 cm), but height declined sharply at 50 mM (9.58 cm) and 100 mM NaCl (4.74 cm). Microalgae showed the steepest height reductions, while SD 008 maintained moderate performance with smaller declines. Root length was longest with microalgae at 50 mM (7.40 cm) and with SD 027 at 100 mM (5.8 cm). Root count decreased with increasing salinity, but SD 027 and Actino + Microalgae were more effective in maintaining root growth than the control. SD 027 also increased leaf numbers by 30% under 50 mM NaCl, outperforming other treatments. Rice biomass weight showed a significant decrease at 100 mM NaCl concentration, although strain SD 027 was able to increase biomass at 50 mM concentration (0.080 g). This finding highlights the significant impact of salinity stress, but strain SD 027 has the effect of mitigating the negative impacts, especially at moderate salinity levels.

All the treatments were applied, and a consortium of microalgae and actinomycetes were proven to be the most effective in mitigating the negative impact of salinity on germination. Seeds treated with the consortium showed significantly higher germination

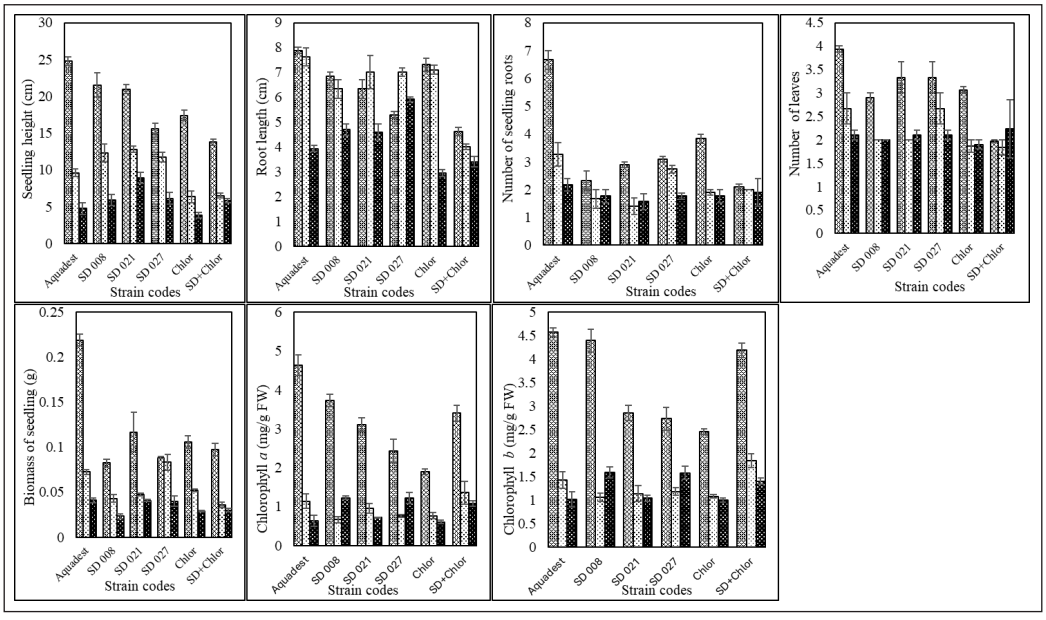

Figure 6. Growth response of 14-day-old In Vitro rice seedlings under salinity stress with Actinomycetes and microalgae treatments

Table 4
Response of 14-day-old in vitro rice seedlings under salinity stress with actinomycetes and microalgae treatments

Treatment	NaCl (mM)	Seedling Height	Root Length	No. of Roots	No. of Leaves	Biomass	Chl a (mg/g fw)	Chl b (mg/g fw)
Aquadest	0	24.786±0.996^{a}	7.881±0.219^{a}	6.666±0.577^{a}	3.933±0.115^{a}	0.219±0.011^{a}	4.630±0.459^{d}	4.570±0.165^{b}
	50	9.583±0.895^{c}	7.631±0.620^{a}	3.266±0.750^{b}	2.666±0.577^{a}	0.072±0.005^{b}	1.137±0.318^{a}	1.423±0.315^{a}
	100	4.743±1.321^{d}	3.933±0.231^{b}	2.166±0.378^{b}	2.101±0.173^{a}	0.041±0.004^{c}	0.637±0.244^{a}	1.513±0.480^{a}
SD 008	0	21.533±2.837^{a}	6.836±0.280^{a}	2.333±0.577^{b}	2.901±0.173^{a}	0.082±0.006^{b}	3.723±0.280^{c}	4.393±0.414^{b}
	50	12.311±2.089^{b}	6.333±6.333^{a}	1.666±0.577^{b}	2.000±0.000^{a}	0.043±0.007^{c}	0.663±0.143^{a}	1.060±0.156^{a}
	100	5.883±1.3194^{d}	4.691±0.420^{b}	1.766±0.404^{b}	2.000±0.000^{a}	0.023±0.005^{c}	0.441±0.545^{a}	0.936±0.733^{a}
SD 021	0	20.983±0.993^{a}	6.333±0.665^{a}	2.901±0.173^{b}	3.333±0.577^{a}	0.116±0.037^{a}	3.107± 0.304^{c}	2.860±0.280^{a}
	50	12.793±0.718^{b}	7.016±1.167^{a}	1.401±0.529^{b}	2.000±0.000^{a}	0.047±0.002^{c}	0.950±0.219^{a}	1.133±0.290^{a}
	100	8.903±1.305^{c}	4.593±0.568^{b}	1.566±0.513^{b}	2.101±0.173^{a}	0.041±0.002^{c}	0.716±0.021^{a}	1.037±0.110^{a}
SD 027	0	15.600±1.241^{b}	5.281±0.235^{a}	3.111±0.173^{b}	3.333±0.577^{a}	0.088±0.001^{b}	2.437±0.525^{b}	2.733±0.418^{a}
	50	11.726±1.121^{c}	7.001±0.291^{a}	2.733±0.251^{b}	2.666±0.577^{a}	0.083±0.015^{b}	0.757±0.065^{a}	1.180±0.131^{a}
	100	6.136±1.354^{d}	5.923±0.170^{a}	1.766±0.208^{b}	2.101±0.173^{a}	0.041±0.011^{c}	0.176±0.025^{a}	1.033±0.072^{a}
Chlorella sp.	0	17.413±1.160^{b}	7.303±0.460^{a}	3.833±0.288^{b}	3.066±0.115^{a}	0.105±0.013^{a}	1.900±0.121^{a}	2.453±0.097^{a}
	50	6.340±1.421^{d}	7.096±0.315^{a}	1.901±0.173^{b}	1.866±0.231^{b}	0.052±0.002^{c}	0.767±0.132^{a}	1.077±0.055^{a}
	100	3.851±0.705^{d}	2.943±0.241^{b}	1.766±0.404^{b}	1.9±0.173^{b}	0.028±0.001^{c}	0.607±0.083^{a}	1.000±0.120^{a}
SD + Chlor	0	13.763±0.755^{b}	4.631±0.290^{b}	2.101±0.173^{b}	1.966±0.057^{b}	0.197±0.012^{a}	1.913±0. 976^{a}	2.190±0.151^{a}
	50	10.591±0.341^{b}	4.011±0.196^{b}	2.000±0.011^{b}	1.833±0.288^{b}	0.035±0.005^{c}	1.357±0.515^{a}	1.840±0.256^{b}
	100	5.836±0.438d	3.401±0.355^{b}	1.901±0.854^{b}	2.233±1.078^{a}	0.031±0.003^{c}	1.801±0.347^{b}	1.400±0.072^{a}

Data represent the mean values of three replicates ±SD. Different letters indicate a significant difference according to Tukey's post-hoc test at $p<0.05$

rates than seeds treated solely with microalgae, indicating a synergistic interaction between the two microorganisms in reducing salt stress. Microalgae were known to support plant growth by increasing photosynthetic efficiency and providing essential nutrients (García-González & Sommerfeld, 2016). In this study, the presence of actinomycetes in the consortium appeared to strengthen this effect, allowing the plant's stress tolerance mechanisms to function more optimally. Salinity stress has been shown to significantly affect rice growth through several mechanisms, such as disrupting water uptake, increasing plant growth, inducing ion toxicity, and producing oxidative stress (Chieb & Gachomo, 2023; Kumawat et al., 2022; Ragaey et al., 2022). A significant decrease in seedling height and root number correlated with increasing salinity. This is consistent with previous findings that salinity disrupts cell elongation and division (Abd El-Hameid & Sadak, 2020; Rodríguez et al., 2023). Treatment with distilled water (control) demonstrated the ability of plants to maintain optimal growth conditions at 0 mM NaCl but was unable to reduce the effects of salt stress at higher concentrations.

Treatment with strain SD 027 appears to have demonstrated its potential as a promising biostimulant, particularly in maintaining root length and biomass under high salinity conditions. Actinomycetes are known to increase plant tolerance to salinity through several mechanisms, including producing phytohormones, stabilising membrane integrity, and facilitating nutrient uptake (Inbaraj, 2021). Furthermore, the biological activity of strain SD 027 demonstrated a high phosphate solubilisation index and IAA production, potentially contributing to its effectiveness in mitigating the impact of salt stress. Similarly, strain SD 021 exhibited high phosphate solubilisation and IAA production, suggesting its potential role in increasing nutrient availability and stimulating root development (Jiang et al., 2019). Furthermore, strain SD 008, with a moderate phosphate solubilisation index and IAA production, demonstrated moderate performance under salinity stress. Importantly, all strains have been demonstrated to have nitrogen fixation capabilities, further supporting their role in nutrient cycling and plant growth under stressful environmental conditions.

Treatment with microalgae showed contrasting results, with a significant decrease in seedling height and biomass at higher salinity levels. This is likely due to the limited capacity of microalgae's bioactive compounds to withstand severe osmotic stress (Martínez-Ruiz et al., 2022). In contrast, the combination of actinomycetes and microalgae has been shown to have a synergistic effect, stabilising root and leaf growth, possibly through increased production of antioxidants and osmoprotectants (Fariza et al., 2024; Sultana et al., 2020). Biomass reduction under salinity stress is a common response in plants, associated with limited photosynthesis and impaired nutrient assimilation (Munns & Gilliham, 2015). In this study, strain SD 027 appeared to mitigate biomass loss, likely by regulating the expression of stress-responsive genes and maintaining cellular homeostasis (Abulfaraja & Jalal, 2021).

The differential response was observed among actinomycete strains at various salinity levels suggest that strain-specific traits play a crucial role in determining plant adaptation to salt stress. Overall, the findings of this study indicate that strains SD 027 and SD 021, as well as the combined application of actinomycetes and microalgae, have significant potential to mitigate the adverse effects of salinity on rice plants. To assess their practical application in agriculture, further research should be directed towards field trials and molecular analysis to better understand the mechanisms underlying the effectiveness of these biostimulants, particularly in soils affected by high salinity.

Chlorophyll Content Analysis

The results of chlorophyll content measurements in rice seedlings treated with microbes and grown at various NaCl concentrations (0, 50, and 100 mM) demonstrated significant physiological changes due to salinity stress. The control treatment with distilled water recorded the highest chlorophyll *a* content (4.6300 mg g^{-1} wet weight), indicating optimal photosynthetic conditions. The response of plants treated with actinomycete strains SD 008 and SD 021 showed lower chlorophyll *a* content compared to the control, but did not differ significantly from each other, while the microalgae treatment produced the lowest value. All treatments at a concentration of 50 mM NaCl showed relatively uniform responses for chlorophyll *a* and *b*, with no significant differences. Interestingly, at 100 mM NaCl, the combination of actinomycetes and microalgae was able to significantly increase chlorophyll *a* content compared to other treatments, while chlorophyll *b* remained relatively constant in all treatments.

Chlorophyll *a* and *b* are essential components in the process of photosynthesis. The concentration levels of these two chlorophylls often indicate the physiological status of plants under environmental stress. Chlorophyll content decreased with increasing NaCl concentration. This corresponds to the inhibitory effect of salinity on photosynthesis, caused by ion imbalance, oxidative stress, and pigment degradation (Hameed et al., 2021; Sadak et al., 2022).

Meanwhile, the 0 mM NaCl treatment (control) showed high chlorophyll *a* levels. This effect is due to the absence of stress factors, allowing chlorophyll synthesis to proceed unimpeded. On the other hand, lower chlorophyll *a* levels were observed in the actinomycete treatments (SD 008 and SD 021). This is related to low physiological adaptation, although the two strains did not differ significantly from each other. Treatments with microalgae exhibited the lowest chlorophyll *a* levels, possibly due to resource competition or suboptimal fitness under these conditions (Kumar et al., 2020; O'Callaghan et al., 2022).

Furthermore, at 50 mM NaCl, there were no significant differences among treatments, suggesting a threshold effect where all treatments experienced a similar degree of stress-

induced decrease in chlorophyll synthesis. This study's findings align with previous research reporting that moderate salinity can induce similar physiological responses in plants (Etesami & Glick, 2020).

The effect of 100 mM NaCl concentration emphasised the potential synergistic effect of the consortium between actinomycete strains and microalgae in reducing high salinity stress. This synergy is thought to occur through complementary mechanisms. Increased nutrient uptake, reduced oxidative damage, or improved osmotic adjustment are mediated by interactions between microbes (Li et al., 2017). The absence of differences in chlorophyll *b* levels across all treatments indicates that salinity stress and microbial effects play a significant role in influencing the chlorophyll *a* biosynthesis pathway or its associated degradation mechanisms.

Overall, our findings highlight the potential application of microbial inoculants, particularly actinomycetes, and their combination to positively maintain chlorophyll levels under salinity stress. Further studies are recommended to explore the molecular and physiological mechanisms underlying these effects to optimize their application in agriculture on saline soils.

Proline is a crucial compound in plant physiological reactions. High proline content is widely used as a biochemical marker reflecting a plant's physiological ability to adapt to environmental stress. Environmental stress, particularly salinity, proline acts as an osmoprotectant and stabilises cellular structures (Dellero et al., 2020; El Moukhtari et al., 2020). Our findings demonstrate differences in rice root responses to NaCl-induced salinity stress under various microbial treatments.

Under saline conditions (0 mM NaCl), relatively high proline levels were observed in the control and SD 021 treatments. This indicates that these conditions support baseline proline synthesis for normal physiological function. Conversely, the significantly lower proline content in the SD 008 treatment indicates low proline biosynthetic activity. Furthermore, more efficient cellular homeostasis mechanisms are established when plants are not exposed to environmental stress. This evidence aligns with previous reports showing that proline accumulation is generally low when plants are not under environmental stress (Hosseinifard et al., 2022).

Meanwhile, at moderate salinity levels (50 mM NaCl), microalgae treatment was observed to stimulate a significant increase in proline accumulation. This is thought to be related to the microalgae's ability to produce extracellular metabolites, thus stimulating plants to overcome osmotic imbalances and maintain stress tolerance (Kishor et al., 2022). Furthermore, treatment with strain SD 008 also showed relatively high proline levels. This indicates its potential to stimulate the proline biosynthesis pathway, a physiological characteristic often found in high-salinity-tolerant actinomycetes (Ahammed et al., 2020; Sarsekeyeva et al., 2024).

Furthermore, in the 100 mM NaCl treatment, increased proline accumulation was found in both the microalgae treatment and the combination of microalgae and actinomycetes. This may indicate a synergistic effect in alleviating high salinity stress. The effectiveness of microalgae is thought to be related to their ability to produce compatible compounds and antioxidant molecules. Meanwhile, actinomycetes are thought to play a role in increasing nutrient uptake and maintaining ion balance within plant cells (Farfan-Cabrera et al., 2022). The opposite effect was observed, with the lowest proline content found in the control treatment under high salinity conditions. Negative effects of salt stress were observed when microbial treatments were not applied to protect plants from these unfavourable conditions. Overall, these results confirm that microbial inoculants play a key role in regulating proline metabolism, particularly in plants experiencing higher salinity stress. Furthermore, the combination of microalgae and actinomycetes is considered a promising approach to increasing plant tolerance to salinity. Therefore, the molecular and biochemical interactions between the two require further investigation in future research.

Proline is a compound that plays a crucial role in plant physiology, functioning as an osmolyte, cytosolic pH regulator, antioxidant, and protein stabiliser (Khattab et al., 2024; Meena et al., 2019). This compound is crucial for plant adaptation mechanisms. It accumulates in plants when exposed to various abiotic stresses, such as salinity, drought, and extreme temperatures (Badr et al., 2025; Zhang et al., 2023). Several studies have shown that proline plays a role in maintaining osmotic balance, protecting cell structures, and reducing damage from oxidative stress (Nivedita et al., 2024). Interactions between microorganisms in consortia, including actinomycetes and microalgae, have also been reported to increase plant tolerance to salinity. Increased proline accumulation, in response to reducing negative effects, is accompanied by improvements in overall plant physiological resilience (Mishra et al., 2021).

Proline also acts as an osmolyte to maintain cell water potential and prevent cellular dehydration under saline conditions. Furthermore, proline's involvement in signalling pathways that regulate plant responses to stress has been widely reported. This compound is known to interact with various proteins and enzymes, thus influencing the expression of genes related to stress tolerance. This is supported by metabolic adjustments to maintain energy balance when plants are exposed to unfavourable environments (Bakhoum et al., 2022; Renzetti et al., 2025). Proline protects against NaCl-induced oxidative damage by suppressing ROS accumulation and reducing lipid peroxidation. A relationship between increased proline accumulation and plant stress resistance has been demonstrated. Therefore, proline is used as a biomarker in plant breeding programs to improve salinity tolerance in various agricultural commodities (Elsawy et al., 2018).

Analysis of Proline Content

The analysis of proline content revealed considerable variation in proline accumulation across different treatments and NaCl concentrations (Table 5). Under non-saline conditions (0 mM NaCl), the control and strain SD 021 recorded the highest proline levels in the roots, reaching 12.3033 and 12.6367 nmol g^{-1} fresh weight, respectively. In contrast, strain SD 008 exhibited the lowest proline concentration, at 2.9033 nmol g^{-1}.

At a moderate salinity level (50 mM NaCl), the microalgae treatment resulted in the highest proline accumulation (51.8633 nmol g^{-1}), which was significantly greater than that observed in the other treatments. A similar pattern was observed at 100 mM NaCl, where the microalgae treatment again showed the highest proline content (62.9933 nmol g^{-1}), followed by strain SD 027 (44.2733 nmol g^{-1}) and strain SD 008 (40.1867 nmol g^{-1}).

In addition, the combined application of microalgae and actinomycetes also led to a marked increase in proline accumulation under high salinity conditions. By contrast, the control exhibited the lowest proline level under the same conditions, with a value of 18.1000 nmol g^{-1}.

Analysis of MDA Content

The MDA (malondialdehyde) concentration increased with increasing salinity stress, indicating that oxidative stress in rice plants was enhanced (Table 6). Under non-saline conditions (0 mM NaCl), no significant differences were observed among the treatments, and the MDA levels were found to be relatively low. The lowest MDA concentration was recorded in strain SD 021, followed by strain SD 027, whereas the highest value was observed in strain SD 008.

Under moderate salinity stress (50 mM NaCl), the lowest MDA level was again recorded in the treatment with strain SD 021. An intermediate level of MDA was detected in the consortium treatment consisting of microalgae and actinomycetes, while a higher value was observed in the distilled water control.

At a higher salinity level (100 mM NaCl), clearer differences among the treatments were observed. Lower MDA levels were maintained in treatments with strains SD 008 and SD 021 compared with the distilled water control and the microalgae-actinomycetes consortium, in which the highest MDA concentrations were recorded.

Overall, the accumulation of MDA was consistently suppressed by strain SD 021 across different salinity levels, indicating that its potential role in reducing oxidative damage in rice plants can be suggested.

MDA is a compound widely recognised as a reliable indicator for detecting lipid peroxidation. The high accumulation of this compound in plant tissues is thought to reflect the extent of oxidative damage to cell membranes caused by abiotic stress (Sadak & Ramadan, 2021; Yu et al., 2020). Increased MDA levels were observed at higher NaCl

Table 5
In vitro proline content in roots and leaves of 14-day-old rice seedlings under salinity levels (0, 50, and 100 mM NaCl) with actinomycetes and microalgae treatments

Treatment	Proline Content (nmol g^{-1} Fresh Weight)					
	0 mM NaCl		50 mM NaCl		100 mM NaCl	
	Leaf	Root	Leaf	Root	Leaf	Root
Aquadest	10.403±1.130^{c}	12.303±0.915^{c}	14.393±1.955^{b}	33.080±2.0267^{c}	18.100±0.978^{a}	48.257±0.955^{b}
SD 008	12.273±2.059^{c}	2.903±0.688^{a}	20.173±2.045^{c}	26.480±0.6155^{b}	40.187±2.007^{e}	46.653±2.956^{b}
SD 021	3.640±0.605^{a}	12.637±1.708^{c}	18.247±2.639^{c}	20.990±1.9507^{a}	22.520±2.092^{b}	25.100±1.462^{a}
SD 027	6.413±1.047^{b}	5.543±0.783^{b}	21.340±1.262^{c}	26.103±0.773^{b}	44.273±1.046^{d}	48.213±0.607^{b}
Chlorella sp.	3.187±0.985^{a}	5.660±0.406^{b}	4.060±1.1191^{a}	51.863±2.590^{e}	34.140±1.985^{c}	62.993±2.482^{c}
SD+*Chlorella* sp.	14.1767±1.161^{d}	5.704±0.764^{b}	20.197±1.970^{c}	39.127±1.361^{d}	39.793±3.484^{e}	49.297±0.861^{b}

Note. Data represent the mean values of three replicates ±SD. Different letters indicate a significant difference in proline content according to Tukey's post-hoc test at $p<0.05$

Table 6
In vitro MDA content in roots and leaves of 14-day-old rice seedlings under salinity levels (0, 50, and 100 mM NaCl) with actinomycetes and microalgae treatments

Treatment	MDA content × 10^{-5} (nmol g^{-1} fresh weight)					
	0 mM NaCl		50 mM NaCl		100 mM NaCl	
	Leaf	Root	Leaf	Root	Leaf	Root
Aquadest	3.72±0.62^{a}	1.60±0.12^{a}	2.49±0.54^{c}	5.73±0.10^{c}	19.60±4.00^{a}	7.23±0.42^{b}
SD 008	5.61±0.80^{a}	1.88±0.68^{a}	7.74±0.09^{a}	9.73±0.95^{d}	6.44±0.87^{c}	9.93±0.81^{c}
SD 021	3.57±0.15^{a}	1.91±0.01^{a}	8.19±0.36^{a}	1.33±0.040^{a}	8.39±0.63^{c}	4.89±0.30^{a}
SD 027	3.42±0.37^{a}	1.97±0.54^{a}	3.87±0.58^{a}	4.00±0.13^{b}	7.55±0.58^{c}	4.47±0.46^{a}
Chlorella sp.	1.88±0.76^{b}	2.77±0.15^{a}	1.34±0.29^{b}	5.71±0.13^{c}	14.17±0.52^{b}	5.25±0.80^{a}
SD+*Chlorella* sp.	1.91±0.61^{b}	2.86±0.12^{a}	4.85±0.11^{a}	5.31±0.42^{c}	9.00±0.96^{c}	9.23±0.38^{c}

Note. Data represent the mean values of three replicates ±SD. Different letters indicate a significant difference in MDA content according to Tukey's post-hoc test at $p<0$

concentrations, and this trend is consistent with previous reports. Salinity stress can be associated with increased formation of reactive oxygen species (ROS), ultimately resulting in cell membrane damage (Narsing Rao et al., 2022).

In this study, consistently lower MDA levels were recorded in plants treated with strain SD 021, particularly at NaCl concentrations of 50 mM and 100 mM. These results indicate that this strain can be considered effective in reducing oxidative stress in plants. This effect is likely related to increased activity of antioxidant enzymes or production of osmoprotective compounds that may be induced by the SD 021 strain. This is in accordance with the results of studies that have been reported to contribute to increased plant tolerance under various stress conditions (Hegazy et al., 2023).

Interestingly, lower MDA concentrations were also observed in the microalgae-actinomycete consortium treatment compared to the control at 50 mM NaCl. However, its effectiveness was decreased at higher salinity levels, namely 100 mM NaCl. Microalgae are known to produce various bioactive compounds that play a role in increasing plant tolerance to stress. However, their effectiveness can be reduced at very high salinity levels, possibly due to limitations in maintaining cellular metabolic balance (Hamouda et al., 2022).

In contrast, MDA levels were maintained at a moderate range in plants treated with strain SD 027 across all salinity treatments, indicating that this strain can be considered to have a moderate capacity to suppress oxidative damage in plants.

Higher MDA concentrations were observed in plants treated with water under high salinity conditions, indicating the negative effects of uncontrolled ROS formation on plant growth and development. This study highlights the importance of selecting microbial treatments that are characterised by strong antioxidant properties to mitigate oxidative damage caused by stress. The ability of strain SD 021 to maintain lower MDA levels, even under high salinity conditions, was demonstrated in this study. These observations indicate that SD 021 can be considered a promising bioinoculant to improve the tolerance of rice plants to salt stress.

The findings obtained in this study indicate that a marine microbial consortium has the potential to be applied as a promising and environmentally friendly biofertiliser for agricultural lands affected by salinity. Although the results of this study were generated under in vitro conditions, a strong basis has been provided for further research. Therefore, further research needs to be conducted under field conditions and supported by molecular analysis to better understand the genetic and biochemical mechanisms involved in plant stress tolerance. In addition, the development of microbial biostimulants derived from locally isolated microorganisms is considered to have great potential to support more sustainable rice cultivation systems. This approach is expected to contribute to maintaining food security, particularly in marginal agricultural environments where salinity stress is common

CONCLUSION

Actinomycetes and microalgae isolated from marine environments have been shown to enhance rice seedling growth and improve plant tolerance to salinity. Oxidative stress levels in plants were reduced, indicating that the microbial treatment can support better physiological adaptation to salinity conditions. Among the tested strains, SD 027 was identified as the most effective under salinity stress.

Our findings suggest that the microbial consortium can be considered a promising and sustainable biostimulant for salinity-affected agricultural lands. However, further investigation under field conditions was needed to validate these findings in field conditions.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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AI DISCLOSURE STATEMENT

The authors hereby declare that in preparing the manuscript, we used artificial intelligence (AI)-based tools for language editing was applied only to improve the clarity and fluency of the text.

This work represents the intellectual contributions of the authors and was prepared in accordance with the ethical standards of the journal.

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Using Spectral Vegetation Indices to Study the Growth of *Opuntia ficus-indica* and Identify Suitable Production Sites in the Southwestern Region of Saudi Arabia

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ABSTRACT

This study was conducted to investigate the growth of two *Opuntia ficus-indica* varieties (var. *inermis* and var. *Mill*) in eleven geographical sites in the southwestern region of Saudi Arabia. Selected spectral vegetation indices (VIs) were used to identify suitable production sites for each variety. The results showed significant variations between the studied sites for both varieties. The best growth performance of the *inermis* variety was at Thaqeef Canal site, as evidenced by the highest values of the Normalised Difference Water Index (NDWI, 0.477), Normalised Difference Vegetation Index (NDVI, 0.553) and the Green Normalised Difference Vegetation Index (GNDVI, 0.392), and the second highest value of the Soil Adjusted Vegetation Index (SAV, 0.392) compared to other sites. While the moderate growth performance of the var. *Mill* was at the Bani Saad site compared to other sites, as indicated by the values of NDVI (0.575), SAVI (0.428), and GNDVI (0.408). A land suitability analysis focusing on site-variety interactions was conducted using the Analytical Hierarchy Process (AHP). The results indicated that Thaqeef Canal and Al-Shifa were the most suitable sites for the growth of both

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Opuntia ficus-indica varieties. Other sites considered highly suitable for growing the variety *inermis* include Al-hada, Wadi Muharrm and Sayyadah. While Baqran Village, Al-Thumalah farm, and Al-Thumalah were considered highly suitable for growing the variety of *Mill*. The results of this study provide a good basis for future studies aimed at improving agricultural practices and decision-making related to the production of *Opuntia ficus-indica* in the southwestern region of Saudi Arabia.

Keywords: Arid regions, cactus plants, land suitability, spectral reflectance, vegetation indices

INTRODUCTION

Saudi Arabia is characterised by its arid environment and limited water resources, with about 80-90% of the water supply coming from groundwater, which is rapidly depleting and requires proper management (Al-Salamah et al., 2011). As these conditions have exacerbated the problem of desertification and increased sand encroachment on agricultural lands, many challenges have arisen that have led to a shift in plant species in terms of their presence or absence in habitats. The southwestern region of Saudi Arabia encompasses a wide and diverse range of plant species and populations across various ecological niches. Productivity in this region can be enhanced by growing adaptable crops such as prickly pear, an excellent plant for soil conservation and regeneration in arid and semi-arid environments (Al-Aklabi et al., 2016; Osman et al., 2024).

Cactus crops are gaining increasing interest worldwide. In particular, cactus pear (*Opuntia ficus-indica*) can grow on lands where other crops fail due to its unique properties, which provide the ability to withstand harsh conditions, and thus can be used to restore degraded lands (Kumar et al., 2018). Cactus pear has proven its potential as a versatile and sustainable crop in arid and semi-arid regions due to its high-water use efficiency and tolerance to dry conditions (Slot & Fort, 2024). Also, Shoukat et al. (2023) reported that cactus pear is highly valued worldwide for its effective uses in medicine, cosmetics, human nutrition, livestock feed, wastewater treatment, fuel production, and sustainable and environmentally friendly building materials, in addition to its versatility and high environmental adaptability. The phenological, physiological, and structural adaptations enable *Opuntia ficus indica* to maintain reasonable productivity levels in dry rangeland conditions and even under extreme water scarcity (Hassa et al., 2020). In addition, Naorem et al. (2024) noted that the ability of cactus pear to grow in arid environments and poor soils makes it ideal for combating desertification and soil erosion, and it can also be used as livestock feed. Furthermore, its nutritional properties, including antioxidant and antidiabetic effects, enhance its value as a food source. The same has been stated by Sipango et al. (2022) that due to its adaptability to harsh conditions of less fertile soils, high temperatures, and low rainfall, the prickly pear cactus is drought-resistant and well adapted to high temperatures in arid regions, especially on low-profile soils.

Remote sensing plays an important role in the fields of landscape ecology and ecosystem monitoring because it relies on aggregate scales as well as the powerful panoramic view of its spatial and temporal images (Mucher & Hazeu, 2021). In addition, Matyukira and Mhangara (2024) noted that the ability of remote sensing technologies to provide detailed vegetation maps is vital for environmental management and monitoring, where these technologies enable assessment of vegetation status and temporal and spatial changes, providing deep insights into ecosystem dynamics. Nizamani et al. (2024) also noted that GIS and remote sensing technologies have become essential tools for assessing ecosystem services and conserving biodiversity, as they provide valuable spatial and temporal data that enable scientists and decision-makers to monitor and make effective decisions about environmental processes, threats, and conservation strategies. In this context, vegetation indices, derived from remote sensing-based plant canopy data through mathematical algorithms, are simple and highly effective for quantitative and qualitative assessments of vegetation cover, plant vigour, and growth dynamics (Xue & Su, 2017). Therefore, this study aimed to highlight the importance of analysing the growth performance of two *Opuntia ficus-indica* varieties and to identify the appropriate production sites using spectral vegetation indices. More specifically, the study aimed to explore which variety performs best in a given location.

MATERIALS AND METHODS

The Study Area

This study was conducted in the southwestern area of Saudi Arabia, which is considered an important bioclimatic zone for the growth of *Opuntia ficus-indica*. It covers eleven geographical sites (Table 1) in Al-Taif and Maysan Governorates (Al-Ahada, Al-Hadab, Al-Thumalah Farm, Al-Shafa, Bani Saad, Baqran Village, Jabajib, Sayadah, Thaqeef Canal, Al-Thumalah, and Wadi Muharrm), as shown in Figure 1. The southwestern region of Saudi Arabia is characterised by sandy plains, rocky outcrops, valleys, and fallow lands. This region includes a wide range of plant species in a diverse ecological site.

Sampling Strategy

A total of 11 study sites were identified in the southwestern region (Al-Taif and Mayson) of Saudi Arabia using ArcGIS tools "Create Spatial Sampling Locations". The stratified random sampling method was adopted, and the elevation map was used as the input for the stratum (Figure 1). A 5 km x 5 km grid was generated and considered the main plot in the sampled villages/farms (Table 1). For ground truth data and spectral reflectance measurements, subsequently, the main plots were divided into composite plots (1 km x 1 km). A total of 54 composite plots were then identified and further subdivided

into (30 m x 30 m) plots to conduct the study on each composite plot across all study sites (Figure 2). The subplots were further subdivided into smaller subplots (3m x 3m) where the opuntia species were enumerated, and the cladodes were collected for spectral reflectance measurements. A bottom-up approach was adopted in processing the spectral data for land suitability analysis, whereby the unit plot data were upscaled into subplots and composite plots.

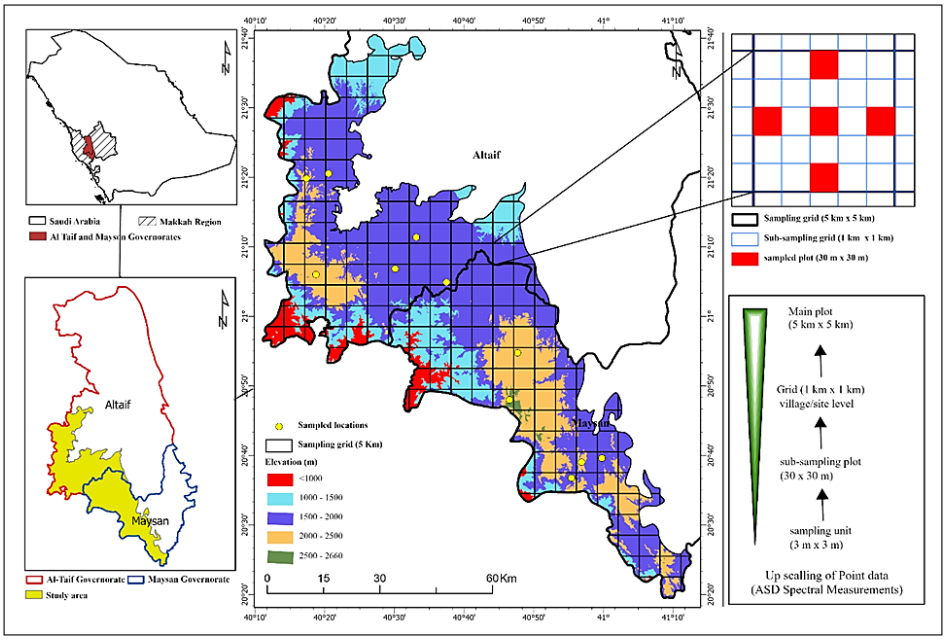

Figure 1. Location map of the study sites

Table 1
*Coordinates of the study sites assigned codes and sampling strategy for cladode collection and spectral data measurements; *no presence of* Opuntia ficus-indica *(L)* Mill

Study site		Longitude	Latitude	Main Plot (5 km x 5 km Grid)	Composite-plot (1 km x 1 km)	Sub-sampled plots (30 m x 30 m)	Unit Plots (3 m x 3 m)
Baqran Village	B	40°37'26.3"E	21°04'53.7"N	1	7	35	175
Bani Saad	CO	40°46'02.4"E	20°53'43.9"N	1	7	35	175
Al-Thumalah Farm	FTH	40°30'05.9"E	21°06'49.0"N	1	4	20	100
Jabajib	G*	40°20'31.3"E	21°20'32.5"N	1	5	25	125

Table 1 (*continued*)

Study site		Longitude	Latitude	Main Plot (5 km x 5 km Grid)	Composite-plot (1 km x 1 km)	Sub-sampled plots (30 m x 30 m)	Unit Plots (3 m x 3 m)
Al-Hadab	HD	40°47'59.1"E	20°46'03.6"N	1	8	40	200
Wadi Muharrm	M*	40°20'09.9"E	21°21'06.2"N	1	1	5	25
Sayadah	SA*	40°59'10.2"E	20°40'12.8"N	1	1	5	25
Al-Ahada	H*	40°17'42.9"E	21°20'35.8"N	1	2	10	50
Al-Shifa	SH	40°20'04.9"E	21°06'21.8"N	1	4	20	100
Al-Thumalah	TH	40°31'11.6"E	21°08'25.5"N	1	7	35	175
Thaqeef Canal	TT	40°55'24.9"E	20°37'10.0"N	1	8	40	200
	Total			11	54	270	1350

Plant Materials and Collection of Spectral Data

From each sampling unit (3m x 3m plot), 3-4 cladodes of *Opuntia ficus-indica* were collected and used for spectral measurements. Three-point measurements of ASD field Spec-3 were performed on the collected cladodes *of Opuntia ficus-indica* varieties (*Opuntia ficus-indica* var. *inermis* and *Opuntia ficus-indica* (L) *Mill*). Spectral reflectance measurements were collected in the laboratory by the FieldSpec3 Spectroradiometer (Analytical Spectral Devices Inc., Longmont, CO, USA) using the direct-contact probe method in a wavelength range of 350-2500 nm. Three spectra were recorded from each cladode, and then the three spectra were averaged to obtain one spectral reflectance curve for each plant sample.

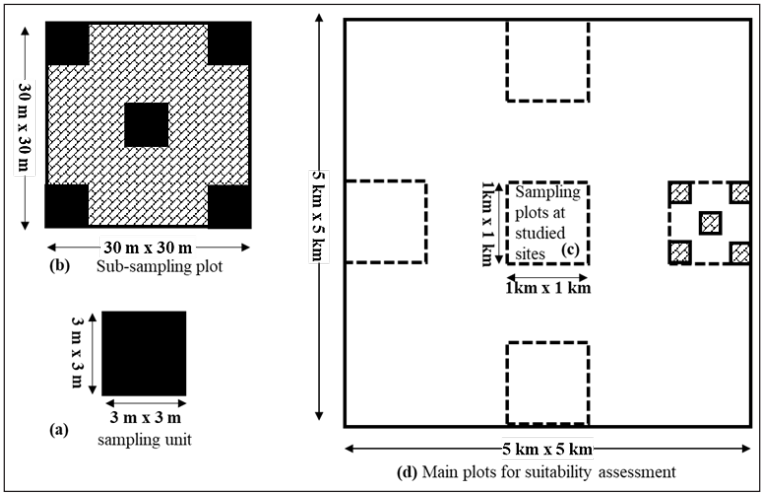

Figure 2. Sampling strategy and plot dimensions for enumeration of *Opuntia* species: (a) sampling unit; (b) sub-sampling plot; (c) sampled plot at study location/village; and (d) main plot for suitability assessment

The collected spectral measurements were then used to calculate four selected vegetation indices (VIs) described in Table 2. The four VIs were adopted to monitor the growth status of cactus pear plants in different studied sites, because they can provide important insights into plants' physiological health, growth patterns, and responses to biotic and abiotic stresses.

Land Suitability Assessment (LSA)

The land suitability assessment (LSA) for the growth of two Opuntia ficus-indica varieties (*O. ficus-indica* var. *inermis* and *O. ficus-indica* (L) *Mill*.) in the southwestern region of Saudi Arabia was carried out by evaluating four selected plant indices (Table 2), which were achieved by analysing laboratory-based collected spectral measurements. The Analytic Hierarchy Process (AHP) is a multi-criteria decision-making method widely used by many researchers for various land suitability models (Robertson & Oinam, 2023). The steps include (i) computation of vegetation indices (VIs), (ii) aggregation - mean values of computed VIs for ecological representativeness and Analytical Hierarchy Process (AHP) weights, (iii) computation of Euclidean distance for assessing spectral-ecological similarity, (iv) computation of Land suitability index (LSI) based on AHP weights (v) execution of Hierarchical Agglomerative Clustering (HAC) for ecologically homogeneous clusters, and (vi) suitability analysis of clusters based on LSI and interpretation.

The processed spectral reflectance data were used to compute key vegetation indices (NDVI, GNDVI, SAVI and NDWI), which represent vegetation greenness, chlorophyll sensitivity, soil adjusted vigour and moisture condition, respectively. ANOVA one-way (species and study sites) and two-way (species x site interaction) analysis was performed for statistical screening and identification of meaningful discrimination among studied parameters using sum of F-statistics and P-value.

Table 2
Description of vegetation indices used for this study

No.	Vegetation Index	Abbreviation	Equation	Source
1	Normalised Difference Vegetation Index	NDVI	$NDVI = \frac{NIR_{800} - Red_{670}}{NIR_{800} + Red_{670}}$	Pettorelli et al. (2005)
2	Normalised Difference Water Index	NDWI	$NDWI = \frac{NIR_{860} - SWIR_{1240}}{NIR_{860} + SWIR_{1240}}$	Gao (1996)
3	Soil Adjusted Vegetation Index	SAVI	$SAVI = \frac{1.5 \times (NIR_{800} - Red_{670})}{(NIR_{800} + Red_{670} + 0.5)}$	Huete (1988)
4	Green Normalised Difference Vegetation Index	GNDVI	$GNDVI = \frac{NIR_{810} - Green_{550}}{NIR_{810} + Green_{550}}$	Candiago et al. (2015)

The AHP analysis, described by Taherdoost (2017), was used for the derivation of weights (%) by pairwise comparison of criteria, normalisation, eigenvector extraction and reciprocal matrix. The pair-wise matrix was computed according to Eq. 1 and Eq. 2, for the studied VIs including the NDWI, NDVI, SAVI, and GNDVI to assess the LSI of *O. ficus-indica* as indirect measures of vegetation health status and water content in the plant cladode. Subsequently, priority Consistency Index (CI) and Consistency Ratio (CR) were also computed.

$$Score_i = F_{species} + F_{site} + F_{interaction} \quad [1]$$

$$Pairwwise\ matrix\ a_{ij} = textScore_i / textScore_j \quad [2]$$

where $score_i$ is the Normalised contribution of the *i-th* index.

Consequently, the LSI was computed from VIs using AHP weights, as per Eq. 3.

$$LSI = w_1 NDVI + w_2 GNDVI + w_3 SAVI + w_4 NDWI \quad [3]$$

Where *w* is the relative importance of a vegetation index; W_i is the AHP weight corresponding to VIs. The obtained LSI ranges are categorised into statistically derived four suitability classes: best (0.75 - 1.00), good (0.50-0.75), fair (0.25-0.50) and not suitable (0.00-0.25).

To identify natural ecological groupings among studied sites and Opuntia varieties (i.e., *Inermis* and *Mill*), HAC was applied using Ward's linkage and Euclidean distance on mean vegetation index values (NDVI, GNDVI, SAVI, and NDWI). The output of the hierarchical structure was visualised as dendrograms (Robertson & Oinam, 2023). Finally, the obtained cluster patterns were interpreted for land suitability with the help of LSI and elevation data across the studied locations and Opuntia varieties (i.e., *Inermis* and *Mill*). The clustering quality was assessed by employing the Silhouette Coefficient and the Davies-Bouldin Index, how well sites are species-grouped into meaningful clusters.

RESULTS AND DISCUSSION

Descriptive Statistics of the Studied Vegetation Indices

The results of vegetation indices for the two studied cactus pear varieties at different experimental sites in the southwestern region of Saudi Arabia are shown in Table 3. These results were analysed for site and variety, and significant differences were found. For example, the *inermis* variety at the TT site showed the highest values of NDWI (0.477),

NDVI (0.553), and GNDVI (0.392), and the second highest value of SAVI (0.392), compared to the other study sites. While the moderate growth performance of the *Mill* variety was observed at the CO site compared to the other study sites, as shown by the highest values of NDVI (0.575), SAVI (0.428), and GNDVI (0.408). Conversely, the HD site showed the lowest values of VIs, indicating poorer vegetation conditions, with var. *inermis* having the lowest values of NDVI (0.394) and SAVI (0.301); and var. *Mill* has the lowest values of SAVI (0.260) and GNDVI (0.180).

To study the distributional characteristics of the combined VIs observations for the two cactus pear varieties at the study sites, additional descriptive statistics were performed by constructing box plots. The box plots, shown in Figure 3, demonstrated that the TT site consistently exhibited higher median values for the studied VIs, indicating better vegetation health in this region. Specifically, at the TT site, the medians of the NDWI, NDVI, SAVI and GNDVI for var. *inermis* were 0.452, 0.536, 0.377, and 0.378, respectively.

Table 3
Descriptive statistics (mean and standard deviation - SD) of vegetation indices

Variety	Location	NDWI		NDVI		SAVI		GNDVI	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
var. *inermis*	B	0.419	0.054	0.399	0.086	0.314	0.059	0.232	0.093
	CO	0.379	0.037	0.465	0.097	0.366	0.073	0.243	0.097
	FTH	0.430	0.027	0.423	0.073	0.334	0.042	0.232	0.097
	G	0.402	0.029	0.407	0.034	0.308	0.027	0.207	0.041
	H	0.445	0.027	0.515	0.039	0.368	0.025	0.334	0.037
	HD	0.433	0.038	0.394	0.083	0.301	0.051	0.242	0.090
	M	0.428	0.020	0.565	0.055	0.406	0.051	0.364	0.061
	SA	0.453	0.023	0.526	0.016	0.365	0.013	0.372	0.022
	SH	0.448	0.032	0.528	0.051	0.374	0.027	0.378	0.041
	TH	0.417	0.032	0.443	0.057	0.343	0.042	0.270	0.065
	TT	0.477	0.053	0.553	0.065	0.392	0.031	0.392	0.064
var. *Mill*	B	0.437	0.035	0.395	0.046	0.299	0.032	0.226	0.039
	CO	0.371	0.007	0.575	0.006	0.428	0.088	0.406	0.007
	FTH	0.470	0.024	0.523	0.027	0.373	0.011	0.302	0.051
	HD	0.399	0.037	0.306	0.080	0.260	0.051	0.180	0.065
	SH	0.454	0.019	0.494	0.033	0.352	0.019	0.353	0.036
	TH	0.407	0.046	0.453	0,065	0.326	0.045	0.288	0.073
	TT	0.426	0.034	0.504	0.036	0.368	0.021	0.367	0.036

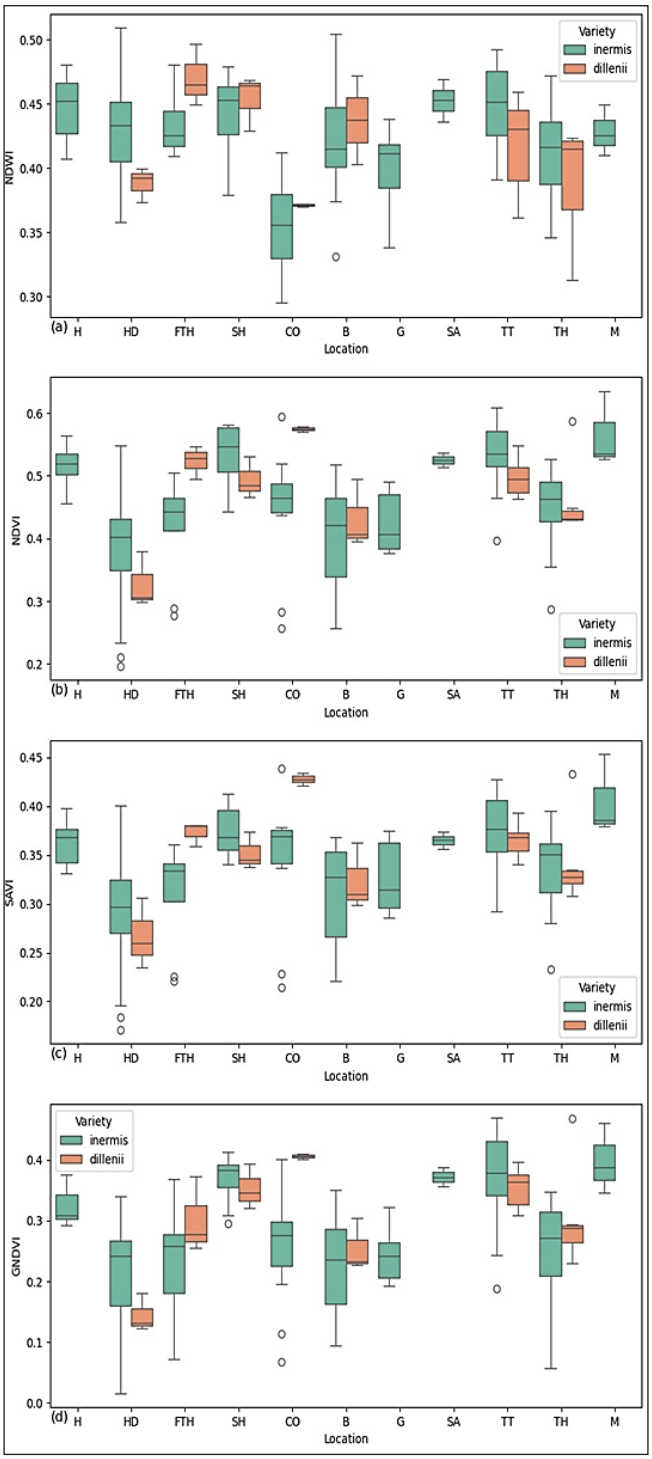

Figure 3. Box plots of the studied vegetation indices on Opuntia species: *O. ficus-indica* var. *inermis* and *O. ficus-indica* (L) var *Mill*

While for var. *Mill*, the medians of the NDWI, NDVI, SAVI and GNDVI at the TT site were 0.431, 0.495, 0.369, and 0.363, respectively. Conversely, the HD site showed the lowest median values, indicating poor vegetation conditions, with var. *inermis* having medians NDWI, NDVI, SAVI and GNDVI of 0.433, 0.403, 0.297, and 0.242, respectively. Meanwhile, the medians of NDWI, NDVI, SAVI and GNDVI at the HD site were 0.392, 0.306, 0.260, and 0.131, respectively.

On average, var. *inermis* outperformed var. *Mill* by recording higher medians of VIs across most study sites, highlighting its robustness and adaptability. In contrast, var. *Mill* recorded lower medians of VIs across the study sites, indicating poor vegetation health. These results underscore the importance of site and variety in influencing vegetation indices, providing valuable insights for improving agricultural practices and developing crop management strategies. Therefore, further analysis focusing on site- and variety-based interactions is necessary.

ANOVA Analysis

The ANOVA analysis showed a stronger statistical effect for NDVI, SAVI, and GNDVI (Table 4). The p-value was below 0.05 (e.g., NDVI site effect p = 0.0165 and GNDVI species effect p = 0.0434), which evidenced a significant effect, whereas higher p-values (such as NDWI species effect p = 0.3634) suggest no meaningful difference. The ANOVA also determined that the GNDVI (p=0.0434) is a significant factor for Opuntia verities. Whereas NDVI, SAVI and GNDVI were found to be significant at the site-level (p values <0.05). Moreover, with respect to species × site combinations, all the studied vegetation indices are significant. ANOVA primarily explained the ecological sensitivity of each studied VIs by measuring how strongly it varies across variety, studied locations, and their interactions. VIs with high F-values and low p-values—such as NDVI, SAVI, and especially GNDVI can be interpreted as stronger discriminatory and greater responsiveness factors.

Table 4
ANOVA results

Index	Variety Effect (F)	Species (p)	Site Effect (F)	Site (p)	Species × Site (F)	Species × Site (p)
NDWI	0.8312	0.3634	1.0671	0.3877	14.9831	<0.001
NDVI	3.2955	0.0715	2.5548	0.0165	5.4369	0.0004
SAVI	2.8689	0.0924	2.0771	0.0496	4.7616	0.0012
GNDVI	4.1494	0.0434	2.4187	0.0226	3.3555	0.0117

Land Suitability - Analytic Hierarchy Process (AHP)

The data-driven pairwise comparison matrix (Table 5) was produced to quantify the relative importance of decision criteria and represented as core input for deriving weights. The pairwise comparison matrix results reveal that GNDVI and NDVI exert the strongest influence on land suitability, followed by SAVI, while NDWI contributes comparatively less. This hierarchy reflects the dominant role of chlorophyll content and vegetation greenness in determining suitability across sites and species.

Furthermore, the eigenvector analysis found that the GNDVI scored the highest weight (0.324) followed by NDVI (0.297), SAVI (0.268) and NDWI (0.115). The final ecological weights for NDWI, NDVI, SAVI and GNDVI were 11.48%, 29.70%, 26.73% and 32.09%, respectively. The validity of weightage (%) was assessed with respect to the obtained CI (2.96×10^{-16}) and CR (3.29×10^{-16}), proven to be internally coherent (CR<0.10). The consistency ratio confirmed the logical reliability of the matrix, supporting the use of derived AHP weights in LSI computation. Among the studied VIs, the GNDVI scored the highest weight (32.09%), reflecting its strong ecological responsiveness to species, site, and interaction effects, while NDVI (29.70%) and SAVI (26.73%) were also influential, representing vegetation greenness and soil-adjusted vigour, respectively. Nevertheless, NDWI was reported with the lowest weight (11.48%), consistent with its low discrimination as detected through ANOVA results.

Higher NDVI and GNDVI threshold (Table 6) values of *inermis* exhibited the best suitability, it explains the performance across a wider index range and establishes greater ecological adaptability. In contrast, the variety of *Mill* shows narrower threshold limits, particularly for GNDVI, higher sensitivity to chlorophyll and site conditions. On the other hand, the obtained threshold values are in agreement with the earlier reported values >0.45, >0.35, >0.35 and >0.40 for NDVI, GNDVI, SAVI and NDWI, respectively (Al-Bakre, 2025; Alexandre et al., 2025).

The land suitability index (LSI) values ranged from 0 to 1, reflecting the combined ecological contribution of each vegetation index using the final AHP weights. These LSI values were subsequently classified into four suitability categories: High (≥0.75), Moderate (0.50-0.75), Fair (0.25-0.50), and Not Suitable (<0.25). Most observations fell within the Moderate class, followed by Fair and Good, indicating that the majority of surveyed locations exhibit intermediate vegetation health influenced by site conditions and species performance. Areas classified as "Highly suitable" correspond to high NDVI and GNDVI values, reflecting vigorous vegetation, while "Fair" and Not Suitable zones were associated with lower values of spectral indices and potential ecological stress. Overall, the LSI results provide a robust, ecologically grounded assessment of land suitability, integrating multiple vegetation indices into a single, interpretable metric for decision-making and spatial planning.

Table 5
AHP-derived pairwise matrix (A) and weights

Criteria	NDVI	GNDVI	SAVI	NDWI	Row Mean (weight)
NDVI	0.296	0.299	0.298	0.296	0.297
GNDVI	0.323	0.325	0.325	0.323	0.324
SAVI	0.266	0.27	0.269	0.266	0.268
NDWI	0.115	0.114	0.116	0.115	0.115

Table 6
AHP derived threshold values for the studied vegetation indices

Vegetation Index	*Inermis*			*Mill*		
	High	Moderate	Fair	High	Moderate	Fair
NDVI	≥ 0.52	0.45 - 0.52	< 0.45	≥ 0.49	0.42 - 0.49	< 0.42
GNDVI	≥ 0.36	0.25 - 0.36	< 0.25	≥ 0.34	0.22 - 0.34	< 0.22
SAVI	≥ 0.37	0.32 - 0.37	< 0.32	≥ 0.35	0.31 - 0.35	< 0.31
NDWI	≥ 0.44	0.40 - 0.44	< 0.40	≥ 0.43	0.41 - 0.43	< 0.41

The Hierarchical dendrogram visualisation and heatmaps of *O. ficus-indica L. f. inermis* (Web) and *O. ficus-indica L.* (Mill) are provided in Figures 4 and 5, respectively. To facilitate the study and discussion of the results, the groups were identified by different colours. The results of the AHP analysis were divided into three distinct groups for the cultivation of *Optunia* Species in the southwestern region of Saudi Arabia: the first group represents the highly suitable sites (green), the second group represents the moderately suitable sites (blue), and the third group represents the relatively suitable sites (red).

The heatmaps and dendrogram provide a visual representation of the hierarchical clusters of *O. ficus-indica* varieties and studied sites, based on the similarities in observed VIs (NDWI, NDVI, SAVI and GNDVI), revealing natural ecological groupings without using suitability weights. The distance between branches indicates the similarity between study sites, where closer locations have relatively similar values of VIs (Figure 4a, Figure 5-a). On the other hand, the heatmaps (Figure 4-b, Figure 5-b) depict the pairwise relationship between VIs in the different study sites for the studied *O. ficus-indica* varieties. The colours of heatmaps indicate the strength of the correlations of VIs, with red representing positive correlations and blue representing negative correlations.

As shown in Figure 4, a detailed analysis of the VIs at different sites for *O. ficus-indica* var. inermis was classified into three clusters. The study sites TT, SH, SA, H, and M were classified as "highly suitable" (green) for *O. ficus-indica* var. inermis production.

While the sites FTH, B, and HD were classified as “moderately suitable” (blue), and the sites TH, G, and CO were classified as “fairly suitable” (red). The inter-cluster linkage distances were set based on the Euclidean distance at 0.2126 (highly suitable to moderately suitable), 0.0698 (moderately suitable to fairly suitable), and 0.1661 (highly suitable to fairly suitable), and the VIs (Figure 4b) facilitate the identification of patterns and relationships between the groups.

In the case of *O. ficus-indica L. Mill,* the dendrogram (Figure 5a) shows the hierarchical clustering of sites based on the NDWI, NDVI, SAVI, and GNDVI. Three clusters were classified as "highly suitable" (green), "moderately suitable" (blue), and "fairly suitable" (red) sites for the growth. The linkage distances between clusters are 0.0388 (highly suitable to moderately suitable), 0.0763 (moderately suitable to fairly suitable), and 0.0794 (highly suitable to fairly suitable). While the heat map (Figure 5b) illustrates the magnitude of the vegetation indices for each site. The colour intensity represents the magnitude of the values, with a colour scale ranging from cool (low values) to warm (high values).

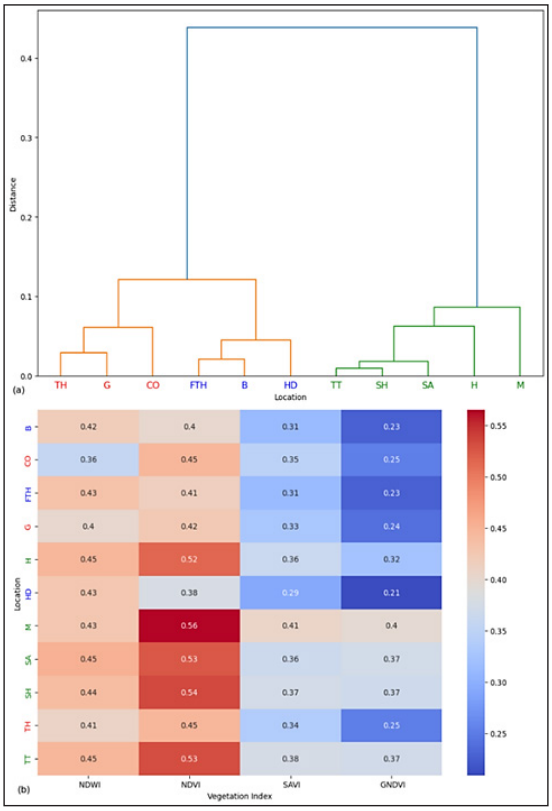

Figure 4. (a) Hierarchical dendrogram classification (AHP); and (b) Heatmap of the studied VIs at different sites for the cultivation of *O. ficus-indica* L. var. *inermis* (web). The sites in the X-axis are marked by green, blue, and red colours, which represent highly suitable, moderately suitable, and fairly suitable, respectively

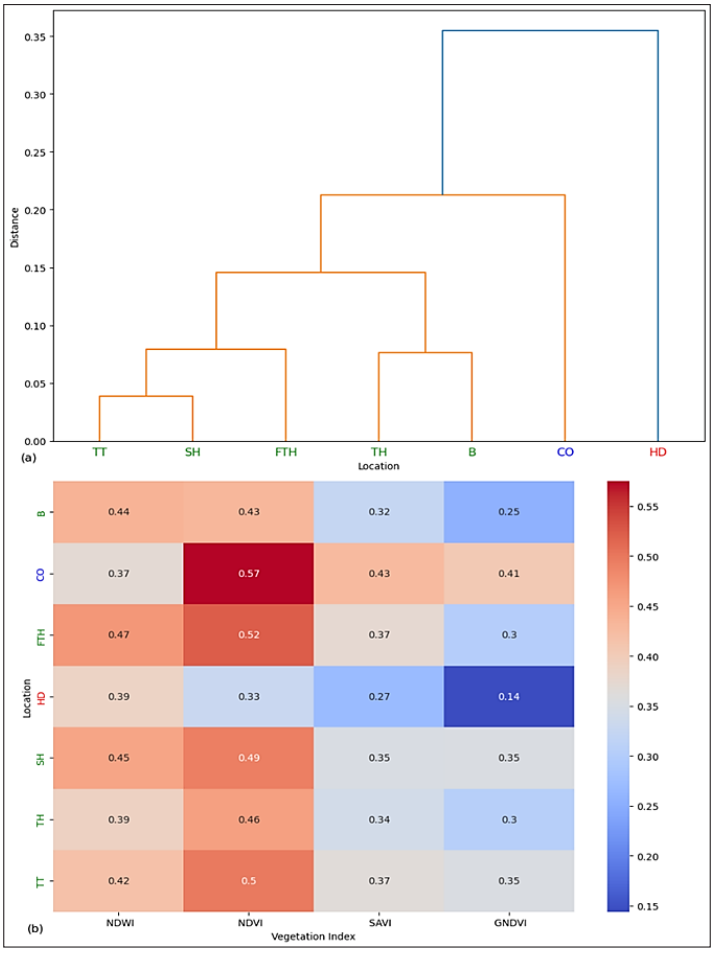

Figure 5. (a) Hierarchical dendrogram classification (AHP); and (b) Heatmap of the studied VIs at different sites for the cultivation of *O. ficus-indica* L. (*Mill*). The sites in the X-axis are marked by green, blue, and red colours, which represent highly suitable, moderately suitable, and fairly suitable, respectively

The mean values of the indices are NDWI (0.418), NDVI (0.473), SAVI (0.350), and GNDVI (0.302). The standard deviations are NDWI (0.037), NDVI (0.078), SAVI (0.049), and GNDVI (0.085), indicating the variability of the indices across sites, facilitating the identification of patterns and relationships within the data. The "highly suitable" group includes sites B, FTH, SH, TH, and TT; the "moderately suitable" group includes site CO; and the "fairly suitable" group includes site HD.

Moreover, the LSI overlaid clusters revealed clear site-dependent suitability patterns for both *inermis* and *Mill* varieties (Table 7). For *inermis*, sites M, SA, SH, and TT were found to be "highly suitable" locations with the highest LSI values (0.70-0.81).

Moderately suitable conditions (0.50-0.70) were observed in sites CO, FTH, G, HD, H, and TH. Whereas, for the variety *mill*, highly suitable sites were TT, SH, and CO, with LSI values ranging between 0.58-0.74, while sites such as TH, FTH, and B showed moderate suitability (0.50-0.60). The least suitable site for the *Mill* was HD (LSI = 0.347), indicating ecological constraints affecting its performance. These site Code-based suitability patterns align with the AHP weighting scheme and clustering patterns, confirming strong ecological differentiation across sites.

Across the study area, land suitability assessment provided a distinct representation with respect to the studied *O. ficus-indica* varieties (*inermis* and *Mill*) and site-dependent response to location. Study sites located within the mid-to-high elevation range (~1700-2100m a.s.l.) corresponded with "high suitable sites". Consequently, the elevation alone has no role in suitability performance. *Inermis* found to have broader elevation adaptability and high suitability across both moderate and higher elevations (~1669-2047 m), has proven its greater ecological resistance. However, *O. ficus-indica* var. *Mill* was shown to have highly suitable sites ranging between ~1850m and 2100m elevation ranges. In contrast, study sites with similar elevation ranges often differed in suitability class, confirming that the local site conditions and *O. ficus-indica* varieties and AHP-weighted VIs played a major role in suitability class determination, but not the elevation factor alone. On the other hand, elevation is represented as an influencing ecological component, but not as a controlling factor on *O. ficus-indica* varieties and land suitability across study sites.

Table 7
Land suitability for the growth of O. ficus-indica *based on LSI and clustering analysis*

Site Code	Study Site	Elevation (m a.s.l.)	*Mill*		*Inermis*	
			LSI	Suitability	LSI	Suitability
B	Baqran Village	1749	0.503	Moderate	0.490	Fair
CO	Bani Saad	2278	0.579	High	0.576	Moderate
FTH	Al-Thumalah Farm	1729	0.513	Moderate	0.599	Moderate
G	Jabajib	1823	-	-	0.519	Moderate
HD	Al-Hadab	2105	0.347	Fair	0.516	Moderate
M	Wadi Muharrm	1980	-	-	0.814	High
SA	Sayadah	1669	-	-	0.744	High
H	Al-Ahada	2073	-	-	0.697	Moderate
SH	Al-Shifa	2074	0.68	High	0.780	High
TH	Al-Thumalah	1687	0.555	Moderate	0.575	Moderate
TT	Thaqeef Canal	1856	0.739	High	0.741	High

Accuracy Assessment

The Silhouette Coefficient and Davies-Bouldin Index (DBI) are widely used cluster validity measures that assess how well sites and species-grouped ecological conditions separate into meaningful clusters. In this study, hierarchical clustering (Ward's method) was applied both site-wise and species × site-wise, producing compact, interpretable groupings of vegetation performance based on GNDVI, NDVI, SAVI, and NDWI responses. The site-wise clustering showed a strong Silhouette score (0.5258) and a low DBI (0.3571), indicating well-separated and compact clusters among study sites, which differ in vegetation structure and ecological condition with high clarity. In contrast, the species × site clustering produced a moderate Silhouette value (0.3572) and higher DBI (0.5569), reflecting more overlap and weaker cluster separation, consistent with the ecological interaction between species and environmental gradients. Overall, these indices confirm that site-level ecological differences are more distinct than species × site interactions, reinforcing the validity of AHP-driven suitability patterns.

Limitations of the Study

In view of the hyper-arid conditions of the study area, where the desert vegetation, such as *Opuntia* spp., is can survive in harsh temperatures and low rainfall. This study was limited to the most influential variables at the landscape scale, i.e., physiological performance-based vegetation indices (NDWI, SAVI, GNDVI and NDVI). Land suitability was performed as described by Robertson and Oinam (2023). This systematic approach enables accurate land assessment to improve Opuntia sp. production, particularly (*O. ficus-indica* (L) *Mill* and *O. ficus-indica* var. *inermis*), while ensuring sustainable agroforestry and horticultural practices. Moreover, *Opuntia* spp. are characterised as high water-use efficiency with stress-tolerant plants under prolonged drought conditions, hence moisture-related or red-edge spectral indices were not studied as those are low-influential parameters at the landscape scale (Louhaichi et al., 2015).

CONCLUSION

This study was conducted to investigate the growth of two *Opuntia ficus-indica* varieties (var. *inermis* and var. *Mill*) in eleven geographical sites located in the southwestern region of Saudi Arabia. The main objective was to identify suitable production sites using spectral vegetation indices (VIs), and to determine the most performing variety in each site. Based on the results of VIs, the Thaqeef Canal site consistently exhibited higher values compared to other sites, indicating better vegetation health in this site. However, among *Opuntia ficus-indica* varieties, the results of VIs indicated that the var. *inermis* outperformed the var. *Mill* by recording higher values of VIs in most study sites. Additional analysis

focusing on site- and cultivar-based interactions was carried out using the Analytical Hierarchy Process (AHP). The results of the AHP indicated that Thaqeef Canal and Al-Shifa were the most suitable sites for the growth of both *Opuntia ficus-indica* varieties. Other sites considered highly suitable for growing the var *inermis* were Wadi Muharrm and Sayadah. While Baqran Village, Al-Thumalah farm, and Al-Thumalah were considered highly suitable for the growth of the var. *Mill*. The results of this study provide a good basis for subsequent studies aimed at improving agricultural practices and decision-making related to the production of *Opuntia ficus-indica* in the southwestern region of Saudi Arabia.

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LIST OF ABBREVIATIONS

H	:	Al-Ahada
HD	:	Al-Hadab
FTH	:	Al-Thumalah Farm
SH	:	Al-Shifa
CO	:	Bani Saad
B	:	Baqran Village
G	:	Jabajib
SA	:	Sayadah
TT	:	Thaqeef Canal
TH	:	Al-Thumalah
M	:	Wadi Muharrm
NDVI	:	Normalised Difference Vegetation Index
NDWI	:	Normalised Difference Water Index
SAVI	:	Soil Adjusted Vegetation Index
GNDVI	:	Green Normalised Difference Vegetation Index
LSA	:	Land Suitability Assessment
VIs	:	Vegetation Indices
AHP	:	Analytical Hierarchy Process
MDPI	:	Multidisciplinary Digital Publishing Institute
DOAJ	:	Directory of Open Access Journals
TLA	:	Three-letter Acronyms
LD	:	Linear Dichroism

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Early Performance Results of Polyhalite on Newly Planted Oil Palms

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ABSTRACT

In oil palm plantations, the application of secondary nutrients, especially calcium and sulfur is often negligible due to the focus on merely primary nutrients and magnesium. Calcium is responsible for proper plant cell division and for strengthening cell walls while sulfur improves the oil synthesis rate in oil palm. Polyhalite ($K_2SO_4 \cdot MgSO_4 \cdot 2CaSO_4 \cdot 2H_2O$) is a natural marine evaporite mineral produced by successive marine evaporation events throughout history. Its huge potential as a potassium fertiliser, due to its very low quantities of chlorine but richness in potassium, calcium, and magnesium, has attracted its use in various crops to achieve higher yields and better-quality crops. In addition to potassium, magnesium fertilisation can increase crop yield and quality. Previous studies focus on applying Polyhalite in cash crops and are less prominent in the oil palm. Therefore, this study aims to evaluate the vegetative growth performance of Polyhalite on oil palm aged one-year-old in addition to the conventional programme. Results showed that applying 0.3 kg ERP + 0.3 kg AgCOTE 18/6/8/2+ME + 0.3 kg Polyhalite into planting holes during transplanting the oil palm seedlings in the field is most cost-efficient while delivering the same vegetative growth compared to the conventional practices.

Keywords: Economic, low carbon, nutrient use efficiency, polysulphate, sustainable

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INTRODUCTION

The labour shortage has been an issue in the oil palm industry for years and has remained uncertain till now. Fewer local people are willing to work in oil palm estates as general workers because they cannot tolerate with the tough working environment in the estate (Azahar, 2022), leading to higher demand for foreign workers to cope with the labour

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requirement. The ratio of workers to plantation area is one worker to 10 hectares of oil palm area (Jasni & Othman, 2017). However, foreign workers coming to Malaysia are mainly employed in the manufacturing and service sectors due to the nature of the working environment, leading to a labour shortage of approximately 40,000 workers in the country's oil palm industry (Evanson, 2024). For instance, the export earnings for Malaysia would reduce to RM10 billion if the number of foreign workers were reduced by 30% (Mamat, 2010). Therefore, the big player in each organisation should look into another approach to deal with the labour shortage issue.

One of the solutions to reduce the impact of the labour shortage issue is to reduce labour requirements through reducing fertiliser application rounds. Thus, controlled-release fertiliser (CRF) is introduced to the market by developing controlled release of nutrients for oil palm seedlings and first-year oil palms. During the immature stage, heavy pest and disease control are required due to the higher vulnerability of the trees. The application of CRF for the first year of oil palm is 1 -2 times per year, depending on the release period of the fertiliser used. Compared to the conventional fertiliser programme of 4 -5 application rounds per year (Goh et al., 2013; SK Specialties, 2020), the reduction in the application rounds can save on labour and handling. In addition, the controlled release system can reduce the losses of fertiliser, reduce the amounts of fertiliser used and minimise environmental pollution (Bah et al., 2014; Jariwala et al., 2022), because the gradual nutrient release from CRF around the root zone can maximise nutrient absorption. Using CRF also enhances economic benefit by 5.21-11.44% (Lyu et al., 2021). Thus, the CRF application can save on labour while at the same time improving the efficiency of nutrient uptake, and this is such a win-win situation for the oil palm industry.

In contrast, the high cost of CRF has turned it into a barrier in the oil palm industry. Price of CRF is usually triple of the price of the compound fertiliser due to its complex coating processing (Govil et al., 2024; Monsouri et al., 2023). As a mature oil palm is a heavy fertiliser feeder which requires at least 10.75 kg/tree/year to sustain 30 mt fruit bunches per hectare (Ng et al., 1999), application of CRF is only applicable during the nursery stage and replanting stage due to insufficient nutrient supply for its aggressive growth. Besides that, polymer-coated CRF poses potential microplastic pollution, which tends to release tiny plastic particles into the environment when the polymer coatings break down in the soil (Stan, 2025).

Besides CRF, Polyhalite is another potential slow-release type of fertiliser source which can be applied to the rooting zone without scorching issues. Polyhalite ($K_2SO_4 \cdot MgSO_4 \cdot 2CaSO_4 \cdot 2H_2O$), or known as Polysulphate in Malaysia, is a natural marine evaporite mineral produced by successive marine evaporation events throughout history. Its huge potential as a K fertiliser due to its very low quantities of chlorine (Cl) but rich in potassium (K), calcium (Ca), and magnesium (Mg) has attracted its use in various crops to

achieve higher yield and better-quality crop. In addition to K, Mg fertilisation can increase crop yield and quality. Ca is also a necessary nutrient for oil palm and can increase root growth, while sulfur helps to improve the oil synthesis. Proper nutrient management is critical for optimising yield and ensuring sustainable cultivation. Thus, sustainable nutrient management practices incorporating these elements are essential for maintaining the long-term viability of oil palm plantations.

Polyhalite is gaining attention as a multi-nutrient fertiliser that enhances crop productivity and soil fertility. Research studies across different crops have demonstrated its potential to improve vegetative growth, yield, and nutrient uptake due to its ability to supply essential secondary nutrients (Ca, Mg, and S) alongside K makes it a promising alternative to conventional fertilisers. There are studies reporting the efficacy of Ca in increasing nutrient availability and uptake in oil palm seedlings (Rahman et al., 2007; Shamshuddin et al., 2024), resistance against basal stem rot (Bivi et al., 2016; Nur Sabrina et al., 2011), and improving physiological activity and drought resistance in oil palm seedlings (Mahyunita et al., 2019). Besides that, sulfur (S) improves soil fertility by enhancing microbial activity and organic matter decomposition, which increases nutrient cycling. Oil palm plantations with adequate S show improved soil organic carbon content and nutrient availability (Sabir et al., 2015).

Previous studies summarised the positive impact of Polyhalite application on various crops, including peanut (Gopinath et al., 2024), maize (Baez-Perez et al., 2021), soybean (Baez-Perez et al., 2021), cabbage (Jamal et al., 2010; Yermiyahu et al., 2017), alfalfa (Bernardi et al., 2018), sunflower (Aziz et al., 2023), potato (Keren-Keiserman et al., 2019), sugarcane (Bhatt et al., 2024), and wheat-based cropping systems (Singh et al., 2023), but there was no study yet reporting on its agronomic efficacy on the immature oil palm trees especially during replanting stage. Additionally, there is limited literature cited on CRF performance reflect conditions specific to tropical oil palm estates. The available commercial trials on the oil palm tree were published on the website, highlighting the economic (Nousbo, 2026) and labour-saving (SK Specialties, 2020) from using CRF. Even though CRF and Polyhalite are both slow-release in nature, their combined use on the growth of oil palm has not been systematically evaluated. Therefore, in this study, we aim to evaluate the early vegetative growth performance of Polyhalite on oil palm aged one year old in addition to the conventional programme. Control treatment without fertiliser application was not included in this study as this is a commercial trial aiming to compare the early performance of palm trees in both agronomic and economic with the incorporation of Polyhalite in addition to the estate's practice. As the estate practice involves the application of rock phosphate, reduced application was also evaluated to determine the optimum dosage and early performance result for both rock phosphate and Polyhalite during transplanting the oil palm seedlings. Besides that, the cost-effectiveness of applying Polyhalite during transplanting in the field was computed to evaluate its economic efficacy.

MATERIALS AND METHODS

The study was conducted for one year, from June 2022 to June 2023, at an oil palm estate located in Keratong, Pahang, Malaysia. The trial size of one (1) hectare was arranged in Randomised Complete Block Design (RCBD) to allocate for three (3) treatments, as shown in Table 1 and *Figure 1*, where each treatment consists of four (4) replicates. While each set of replicates consists of three (3) palms, the total data collection is thus thirty-six (36) palms. One hectare of trial plot is sufficient in an agronomic trial, as the agronomic trials often adopt 3 -4 replicates per treatment as a practical compromise between statistical rigour and resource constraints (Yan, 2021). In perennial crops such as oil palm, estate-scale designs often adopt one-hectare plots with subsampling of 20 -30 palms to measure within-plot variability while remaining operationally feasible (Verdooren, 2019). Throughout the trial period, there was no extreme drought season with two or more consecutive months of low rainfall at below 100mm/month (Table 2). The fertiliser was applied in the planting hole during transplanting, which is a one-time application. Thus, the rainfall variability towards nutrient release is assumed homogeneous across the treatments. The soil samples collected before applying fertiliser treatment confirm the low CEC and low soil nutrient level at the trial site (Table 3). During transplanting, Egyptian Rock Phosphate (ERP) was applied into the planting hole, followed by applying the CRF, AgCOTE 18/6/8/2+ME. Thereafter, the seedlings were placed into the hole and then partially covered with soil. Next, Polyhalite was applied and finally covered the whole seedlings with soil until soil surface. The surrounding soil of the seedlings was well compacted. To align with the estate practice, all immature palm trees were mulched with one whorl of empty fruit bunches. The mulching was conducted at the same time using the same rate across all treatments to minimise potential confounding effects.

The control treatment is the conventional practice in the oil palm industry, whereby 0.5 kg of RP is incorporated into the planting hole together with the CRF, aiming to reduce fertiliser application rounds during the first year of planting, leading to savings in the manuring cost. As both RP and Polyhalite share the function of improving root growth, the reduced dosage for T2 was added to determine the optimum dosage when applying RP and Polyhalite together. The treatment is determined based on the conventional practice as baseline, addition of Polyhalite at the same dosage is included as T3, while a reduced dosage by half for all types of fertiliser is treated as T2. The vegetative parameters include palm height and girth diameter, which were measured on a quarterly basis. Other vegetative parameters, such as the number of green fronds and leaf area, were excluded from this study due to severe beetle attack at the trial plot, which affected the results. Leaf samples of frond #9 were taken twice throughout the trial, at the beginning and at the end of the trial, to compare the leaf nutrient level between the treatments. A study was conducted on testing nutrient prediction on oil palm using spectroscopy, which found that the calibration performance for frond #9, as indicated by R^2 is good for all three nutrients (N, P, K),

respectively, with a relatively low root mean square error of calibration (Jayaselan, et al., 2017). In occasional practices, frond #9 is used for routine leaf sampling for oil palm trees of 3 years and below (Fairhurst et al., 2004).

All vegetative growth data collected were analysed using IBM SPSS Statistics 30.0.0.0. The vegetative parameters were further analysed using repeated-measures ANOVA to test if there were any significant differences among different fertiliser treatments at $\alpha = 0.05$ across the trial period.

Table 1
Description on the treatments in this study

No.	Treatment Number	Type of Fertilisers
1	T1 (control)	0.5kg ERP + 0.5kg AgCOTE 18/6/8/2+ME (AgCOTE)
2	T2	0.3kg ERP + 0.3kg AgCOTE 18/6/8/2+ME + 0.3kg Polyhalite (PS)
3	T3	0.5kg ERP + 0.5kg AgCOTE 18/6/8/2+ME+ 0.5kg Polyhalite (PS)

Table 2
Monthly rainfall at the trial plot throughout the trial period

Year	2022		2023	
	mm	Day	mm	Day
January	505	5	300	17
February	242	17	308	13
March	139	30	511	12
April	383	18	236	20
May	134	8	57	4
June	102	6	200	11
July	54	5	193	9
August	124	8	174	9
September	112	8	216	9
October	319	18	141	13
November	298	18	190	13
December	216	19	254	16
Total	2,628	160	2,780	146

The ideal principle of a CRF should have maximum control of the release so that the nutrients are supplied at a rate similar to the plant growth. Therefore, the estate's practice using AgCOTE was maintained as a control treatment in this study. This fertiliser coated

with the special resin is an epoch-making well controlled releasing high analysis compound coated fertiliser based on nitrate. The release of nutrients from AgCOTE is controlled by the amount of a special chemical release agent in the resin coating. Only the amount of this special release agent varies according to the required period of release, i.e. 360 days. When AgCOTE is placed in the soil, the granules begin to absorb moisture through the coating membrane. This moisture then dissolves the nutrients inside the capsule, which in turn builds up an osmotic pressure. The nutrients can then diffuse through the coating. The amount of release agent contained in the coating will determine how porous the coating is, which determines exactly how fast the nutrients will diffuse.

At the 9th month of the trial period, one round of Royal Terra 15/15/15/2 at 1.50 kg/palm was applied on all treatments due to high temperatures in the tropical region, which shortened the release period of the CRF from 12 months to 9 months. The nutrient release of AgCOTE is moderately affected by temperature. As temperatures warm in the spring and cool in the fall, the release of nutrients will increase or decrease (Figure 2). This keeps the rate of release in tune with the rate of plant growth. Next, the nutrient release of AgCOTE is not significantly affected by soil moisture levels. This release is also unaffected by soil type or soil pH and AgCOTE does not depend upon microbiological decomposition for its action. As the polymer coating is photodegradable, over time, the coating on AgCOTE will be degraded without leaving residues in the soil.

Figure 1. Trial site in Keratong, Pahang, Malaysia

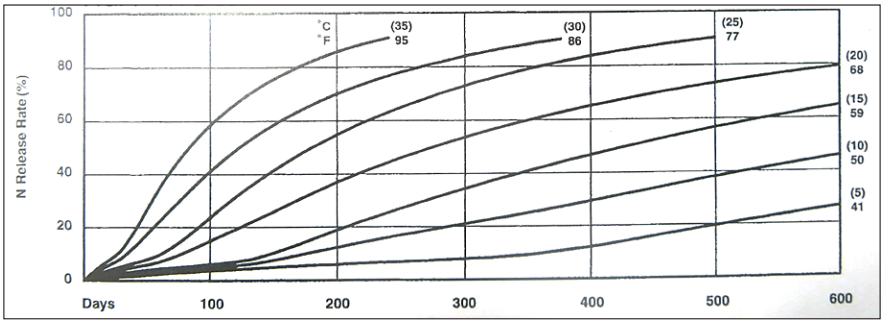

Figure 2. N release rate of AgCOTE at 25 °C, 30°C and 35 °C

Table 3
Soil chemical properties at the trial site

No.	Sampling Depth (m)	Treatment	pH Value	CEC	Organic Carbon	Total N	Phosphorous		Exchangeable Potassium (K)	Exchangeable Magnesium (Mg)	Exchangeable Calcium (Ca)
							Total P	Available P			
				m.e %	%	%	mg/kg	mg/kg	m.e %	m.e %	m.e %
1	0.15	T1	4.48	5.50	0.82	0.17	88	8	0.19	0.21	0.35
		T2	4.87	5.10	1.21	0.22	88	5	0.36	0.24	0.50
		T3	4.66	4.80	0.69	0.11	46	4	0.14	0.15	0.37
2	0.3	T1	4.49	5.00	0.79	0.15	70	6	0.30	0.17	0.27
		T2	4.57	5.40	0.67	0.12	58	4	0.24	0.12	0.32
		T3	4.33	4.70	0.66	0.13	40	3	0.11	0.10	0.31

RESULTS AND DISCUSSION

Normality Test

Normality test results show that the dataset is normally distributed, as the p-value is more than 0.05 based on the Shapiro-Wilk (Table 4). Despite the p-value of 0.041 for the palm height collected for T1 at the 9th Month, the data is still treated as normally distributed due to its minor deviation.

Table 4
Tests of normality using the Shapiro-Wilk

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
Treatment		**Statistic**	**df**	**Sig.**	**Statistic**	**df**	**Sig.**
M0 - Palm Height (cm)	T1	0.210	12	0.151	0.917	12	0.261
	T2	0.244	9	0.130	0.923	9	0.416
	T3	0.180	15	.200*	0.957	15	0.638
M0 - Girth Diameter (mm)	T1	0.202	12	0.192	0.899	12	0.153
	T2	0.169	9	.200*	0.946	9	0.643
	T3	0.138	15	.200*	0.929	15	0.261
M3 - Palm Height (cm)	T1	0.156	12	.200*	0.949	12	0.620
	T2	0.172	9	.200*	0.941	9	0.591
	T3	0.184	15	0.181	0.938	15	0.359
M6 - Palm Height (cm)	T1	0.167	12	.200*	0.923	12	0.315
	T2	0.184	9	.200*	0.906	9	0.287
	T3	0.141	15	.200*	0.955	15	0.605
M6 - Girth Diameter (mm)	T1	0.235	12	0.066	0.894	12	0.134
	T2	0.212	9	.200*	0.927	9	0.449
	T3	0.110	15	.200*	0.961	15	0.718
M9 - Palm Height (cm)	T1	0.201	12	0.196	0.854	12	0.041
	T2	0.236	9	0.159	0.859	9	0.094
	T3	0.167	15	.200*	0.962	15	0.730
M9 - Girth Diameter (mm)	T1	0.203	12	0.186	0.890	12	0.119
	T2	0.167	9	.200*	0.945	9	0.637
	T3	0.130	15	.200*	0.980	15	0.968
M12 - Palm Height (cm)	T1	0.166	12	.200*	0.952	12	0.663
	T2	0.187	9	.200*	0.903	9	0.270
	T3	0.168	15	.200*	0.956	15	0.626

Table 4 (*continued*)

Treatment		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
M12 - Girth Diameter (mm)	T1	0.247	12	0.042	0.886	12	0.105
	T2	0.255	9	0.094	0.898	9	0.243
	T3	0.141	15	.200*	0.954	15	0.582

*. This is a lower bound of the true significance.
a. Lilliefors Significance Correction

Vegetative Growth

Mauchly's test indicated that the assumption of sphericity had been violated for both palm height, at χ^2 (2) = 54.787, $p < 0.001$ (Table 5), and girth diameter, at χ^2 (2) = 11.279, $p = 0.046$ (Table 6). Therefore, degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.515$ for palm height and $\varepsilon = 0.807$ for girth diameter).

Table 5
Mauchly's test of sphericity for palm height

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[b]		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Time	0.175	54.787	9	0.000	0.515	0.583	0.250

Tests the null hypothesis that the error covariance matrix of the orthonormalised transformed dependent variables is proportional to an identity matrix.
a. Design: Intercept + Treatment
Within Subjects Design: Time
b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Table 6
Mauchly's test of sphericity for girth diameter

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[b]		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Time	0.701	11.279	5	0.046	0.807	0.929	0.333

Tests the null hypothesis that the error covariance matrix of the orthonormalised transformed dependent variables is proportional to an identity matrix.
a. Design: Intercept + Treatment
Within Subjects Design: Time
b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Results from Table 7 and Table 8 indicate that the palm height and girth diameter grow significantly across the nine-month period at $p < 0.001$, but different fertilisers did not significantly produce different average palm height and girth diameter at $p = 0.503$ and $p = 0.685$, respectively. The results suggest that the addition of Polyhalite into a conventional manuring program is comparably effective even at reduced dosage.

Table 7
Tests of within-subjects effects for palm height

	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Sphericity Assumed	164897.305	4	41224.326	113.775	0.000
	Greenhouse-Geisser	164897.305	2.060	80046.424	113.775	0.000
	Huynh-Feldt	164897.305	2.332	70702.135	113.775	0.000
	Lower-bound	164897.305	1.000	164897.305	113.775	0.000
Time * Treatment	Sphericity Assumed	2454.743	8	306.843	0.847	0.563
	Greenhouse-Geisser	2454.743	4.120	595.805	0.847	0.503
	Huynh-Feldt	2454.743	4.665	526.254	0.847	0.514
	Lower-bound	2454.743	2.000	1227.372	0.847	0.438
Error (Time)	Sphericity Assumed	47827.690	132	362.331		
	Greenhouse-Geisser	47827.690	67.981	703.548		
	Huynh-Feldt	47827.690	76.965	621.419		
	Lower-bound	47827.690	33.000	1449.324		

Table 8
Tests of within-subjects effects for girth diameter

	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Sphericity Assumed	298236.231	3	99412.077	275.623	0.000
	Greenhouse-Geisser	298236.231	2.422	123146.724	275.623	0.000
	Huynh-Feldt	298236.231	2.786	107056.002	275.623	0.000
	Lower-bound	298236.231	1.000	298236.231	275.623	0.000
Time * Treatment	Sphericity Assumed	1325.482	6	220.914	0.612	0.720
	Greenhouse-Geisser	1325.482	4.844	273.657	0.612	0.685
	Huynh-Feldt	1325.482	5.572	237.900	0.612	0.708
	Lower-bound	1325.482	2.000	662.741	0.612	0.548
Error (Time)	Sphericity Assumed	35707.519	99	360.682		
	Greenhouse-Geisser	35707.519	79.919	446.795		
	Huynh-Feldt	35707.519	91.931	388.415		
	Lower-bound	35707.519	33.000	1082.046		

There was no significant difference in palm growth with the addition of Polyhalite, as shown in Figure 3, Figure 4, and Figure 5. However, palm growth performed slightly better with the addition of 0.3 kg Polyhalite compared to the palm tree without Polyhalite and the palm tree with 0.5 kg Polyhalite. Too high rate of Polyhalite may suppress palm growth because excessive cations supplied are not balanced with the nitrogen and phosphorus. Furthermore, the absence of an immediate morphological response despite improved leaf nutrient concentrations suggests a phase of internal nutrient accumulation. During the first year of planting, immature oil palms prioritise establishing a robust physiological foundation. Increased internal nutrient levels, particularly K and Mg, which may be diverted toward metabolic sink strength and root architecture development rather than immediate above-ground biomass expansion (Mohidin et al., 2019).

Nutrient release rate of Polyhalite demonstrated a prolonged releasing pattern reaching 50 days and constantly delivered nutrients to the tree despite heavy rain following application (Massawe, 2010). According to Huang (2020), Polyhalite's slow-dissolving nature minimises leaching losses during heavy rain by retaining nutrients within the root zone longer than conventional fertilisers. Such a mechanism is particularly suited for

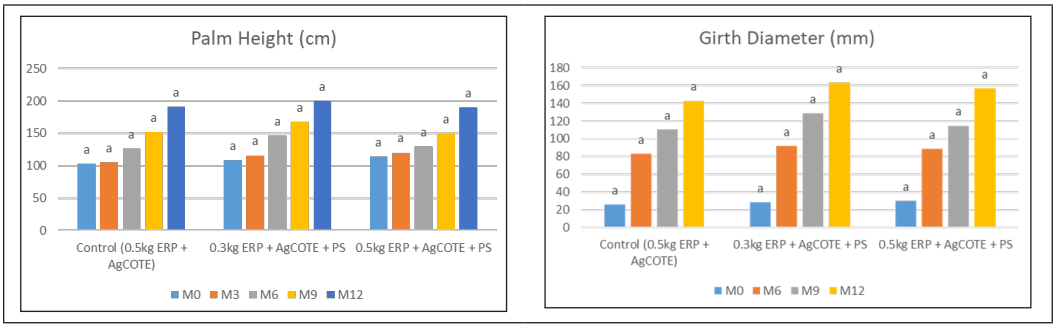

Figure 3. Change in the palm height and girth diameter across different treatments

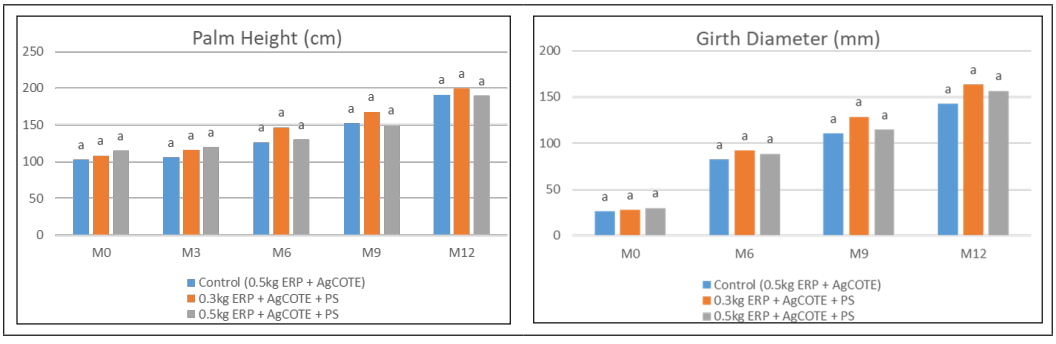

Figure 4. Change in the palm height and girth diameter across the one-year trial period

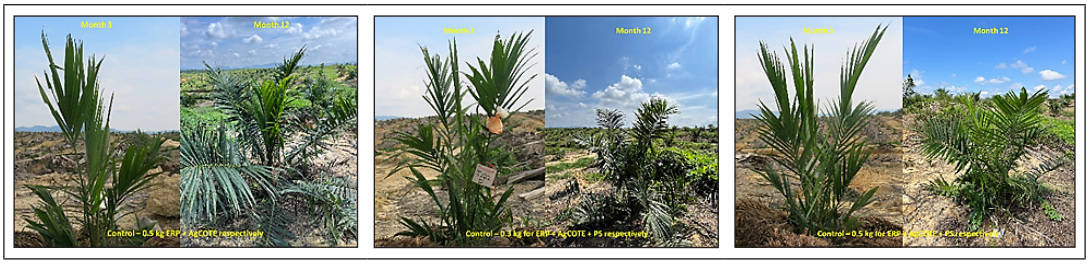

Figure 5. The palm growth progress during month 3 and month 12 after transplanting for (left) control treatment (0.5kg ERP + 0.5kg AgCOTE); (centre) 0.3kg ERP + 0.3kg AgCOTE + 0.3kg PS; (right) 0.5kg ERP + 0.5kg AgCOTE + 0.5kg PS

tropical sites, sustaining vegetative growth by preventing nutrient washout during intense wet periods.

In addition, regular application of calcium minerals on Ultisols and Oxisols maintained soil fertility and improved soil structure. Calcium amendments improved soil aggregation and reduced soil compaction, facilitating better root growth (Shamshuddin et al., 2024). Thus, the addition of 0.3 kg Polyhalite can reduce the dosage of RP and CRF while at the same time achieving a similar growth rate. The application of Polyhalite helps to improve soil structure due to its calcium content, which improves the soil porosity and aggregates soil particles. This helps to reduce erosion, improve water infiltration, and root penetration. In addition, Polyhalite helps to improve soil cation exchange capacity (CEC), which has better nutrient retention and lower nutrient leaching (Huang et al., 2020).

A study found that the partial replacement of potassium chloride (KCl) with Polyhalite improves the soil quality and better growth on peanuts (Tan et al., 2022). Besides, compound fertiliser that contains Polyhalite enhance the yield of winter melon, improves nutrient use efficiency, quality, and reduces environmental pollution (Chen et al., 2022).

A study showed that fertilisers containing Polyhalite increase the content of exchangeable Ca, Mg, and S in the soil. These findings demonstrate the capability of Polyhalite in addressing fertiliser-induced soil acidification, thereby supporting sustainable agricultural practices through enhanced long-term soil health, reduced reliance on chemical amendments, and minimised environmental impacts (Lewis et al., 2019).

Treatment with Polyhalite shows slightly taller palm height, although the difference is not significant, and this could also be due to the contribution of S in Polyhalite. Polyhalite contains 19% of S, the contribution of S helps to enhance leaf area, a higher number of leaves, higher plant height and improve root growth. The enhanced leaf area and plant growth might have supported higher photosynthetic activity, increased N and P uptake, and a higher total dry matter yield, ultimately leading to the higher yields (Li et al., 2025).

Sufficient S boosts nutrient uptake, which is essential for root development, acting as a redox regulator and precursor for compounds vital to root growth. Plant starvation with S disrupts N and S metabolism, leading to reduced fine root length, surface area, and biomass, ultimately impairing root activity and nutrient absorption (Zhou et al., 2024). The abundant Ca ions in Polyhalite actively contribute to cell differentiation at the root tip and root elongation by regulating root morphogenesis through phytochrome mediation and stress signalling in the primary root (Zhang et al., 2020).

Leaf Analysis Results

The leaf analysis results from *Figure 6* show that a higher rate of Polyhalite would deliver higher leaf nutrients. In addition, reduced dosage of RP, Polyhalite and CRF from T2 still delivered equivalent leaf P and leaf Ca levels compared to T1 and T3, which are of higher application dosage. The Ca will usually deposit in root tissues rather than staying on the leaf (Khor et al., 2023). Besides delivering nutrients for plant vegetative growth and nutrient use efficiency, the readiness of Ca plays a role in boosting the plant's immune system against diseases through the Ca deposition in the cell wall for membrane firmness (Khor et al., 2023). The addition of Polyhalite also helps to increase nutrient uptake, especially N, Mg and Ca. This enhanced uptake is supported by the fact that the timing of Polyhalite application aligns with the nutrient demand curves of immature oil palms, which require a steady and continuous supply of K, Mg, and Ca for rapid vegetative development. While conventional high-solubility fertilisers often create a 'peak and trough' nutrient profile that leads to temporary surplus and subsequent leaching, Polyhalite's 50-day release pattern (Massawe, 2010) provides a synchronised nutrient flux. This ensures that nutrient availability matches the palm's constant physiological requirements during its immature phase, particularly in high-rainfall tropical environments.

The treatment with high input of Polyhalite has the highest leaf calcium level after one year. This could be due to the mode of nutrient release and the natural characteristics of Polyhalite. Polyhalite release nutrient through the dissolution of soil moisture. It supplies the nutrients to the crops at a slow and steady rate over a long period, which reduces the risk of nutrient leaching (Tan, et al., 2022). Besides, the dissolution rate can be higher in acidic soil due to the occurrence of hydrogen ions that promote the breakdown of Polyhalite (Singh et al., 2023).

A balanced supply of readily available S through Polyhalite application significantly enhances N uptake in plants, while also fostering synergistic interactions with other essential nutrients. Sulphur augments N assimilation, it facilitates crucial metabolic processes (NO_2^- and SO_4^- reduction), remains fundamental to the amino acid biosynthesis and is for the efficient nutrient translocation within the plant tissues (Singh et al., 2023).

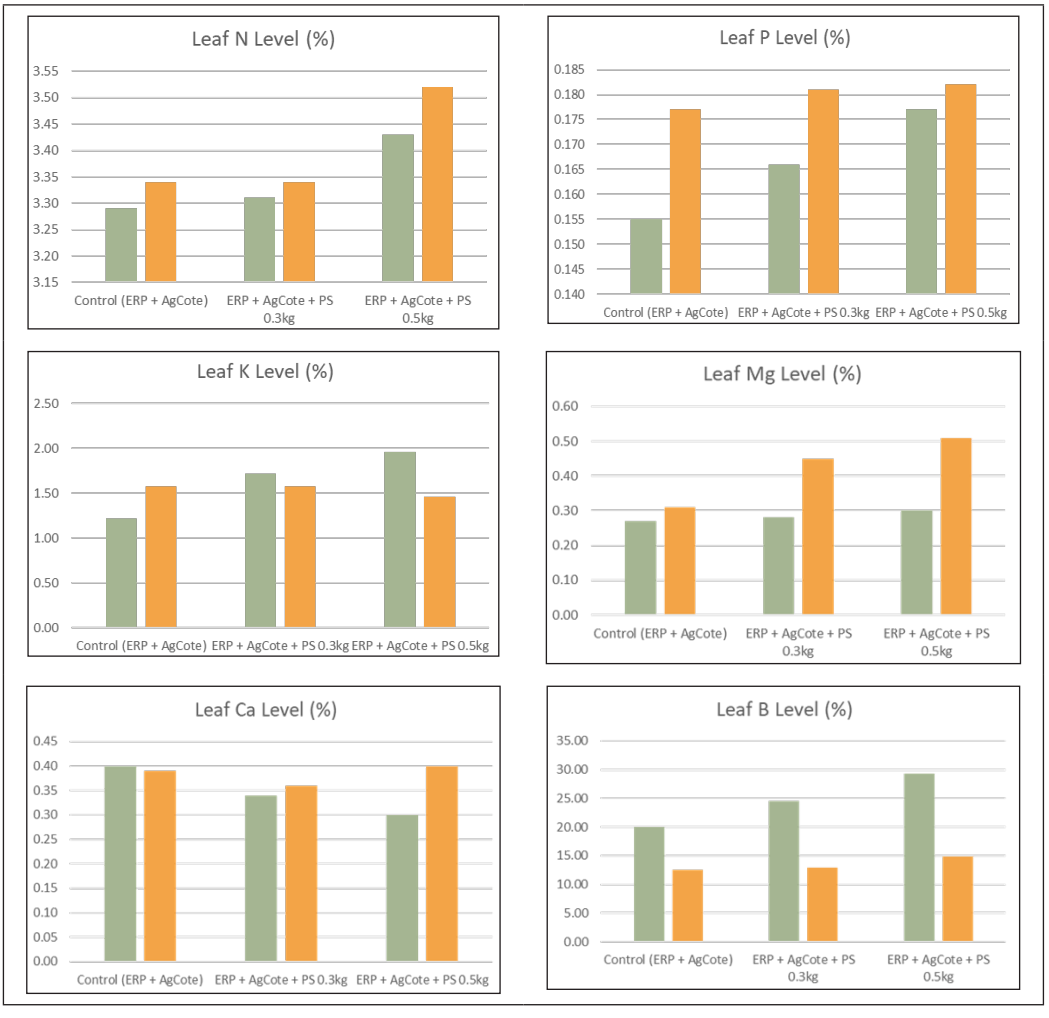

Figure 6. Leaf nutrient level at one year before and after the treatments

Cost Effectiveness

Based on the market price during the trial period, Figure 7 illustrates that the application of 0.3 kg Polyhalite, besides 0.3 kg ERP and 0.3 kg AgCOTE 18/6/8/2+ME, has the lowest fertiliser cost at RM3.05/tree compared to the control treatment (RM4.28/tree) and 0.5 kg treatment (RM5.08/tree). The savings from using 0.3 kg Polyhalite are as high as 40% compared to the control treatment. Each palm can save from RM1.23 by using Polyhalite-combination treatment. Additionally, based on cost sensitivity analysis, every 5% change in the market price of Polyhalite will result in merely 1% change in the total fertiliser cost for the first year of oil palm planting (*Table 9*). The fertiliser price for this study is calculated based on the current market price and varies according to the market.

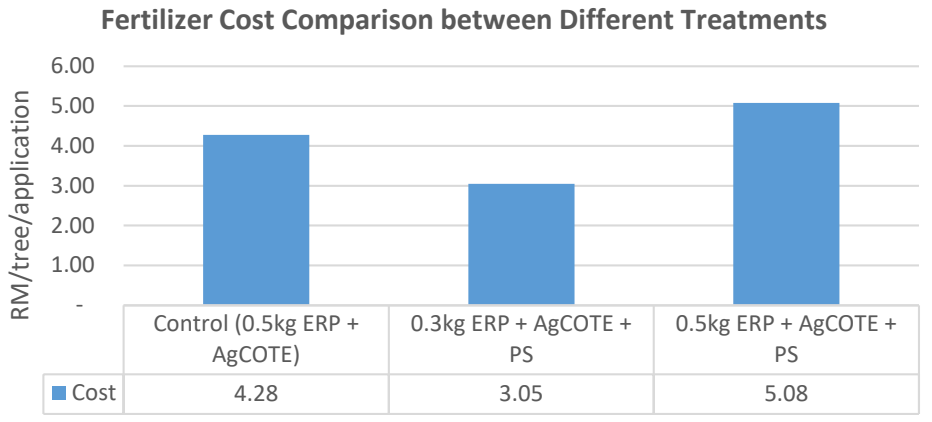

Figure 7. Fertiliser cost comparison between different treatments

Note. ERP -RM550/mt; AgCOTE 18/6/8/2+ME -RM8,000/mt; Polyhalite -RM1,600/mt

Table 9
Cost sensitivity of Polyhalite

No	% Change	Fertiliser Cost (RM/MT)				
		ERP	AgCOTE	Polyhalite	Total Fertiliser Cost	% Increase
1	-10%	550	8,000	1,440	9,990	-2%
2	-5%	550	8,000	1,520	10,070	-1%
3	0%	550	8,000	1,600	10,150	0%
4	5%	550	8,000	1,680	10,230	1%
5	*10%*	550	8,000	1,760	10,310	2%
6	15%	550	8,000	1,840	10,390	2%

The labour savings achieved in this study are distinguished from estate management efficiencies by focusing on the structural design of the manuring program. While the industry's conventional practice (straight fertilisers) requires a high frequency of 4 to 5 rounds (Goh & Härdter, 2003), all treatments in this study utilised CRF technology to consolidate application into fewer rounds. Specifically, T2 achieved additional efficiency by optimising the dosage (0.9 kg/palm) compared to T3 (1.5 kg/palm). This reduces the total weight of material handled and transported by workers, thereby decreasing the man-days required per hectare regardless of the specific labour management protocols of the estate.

Integrated Environmental Assessment

In this study, the integrated use of Polyhalite and polymer-coated CRF represents a dual-strategy for nutrient longevity. However, a comprehensive environmental assessment

must account for the different footprints of these technologies. While the polymer-coated component provides precise control over nutrient release, it introduces synthetic coatings into the soil. Although an empirical assessment of microplastic persistence (Li, 2025) was beyond the technical scope of this trial, the inclusion of Polyhalite serves as a vital sustainability bridge.

As a naturally occurring mineral that dissolves completely into K, Ca, Mg, and S, Polyhalite allows for a reduction in the total volume of synthetic-coated material required per hectare (as seen in the optimised T2 dosage) without compromising the prolonged release pattern (Massawe, 2010). Furthermore, the evidence suggests that the palm's growth was governed by nutrient synchronisation rather than osmotic stress. This is supported by the low salt index of Polyhalite at 0.85 (Yermiyahu, 2017), which, when combined with CRF, maintains a stable soil solution conductivity. By integrating mineral-based slow-release sources with coated technologies, estates can achieve the necessary synchronisation with palm demand curves while effectively reducing the cumulative environmental and plastic burden per application.

CONCLUSION

Additional application of 0.3 kg Polyhalite besides 0.3 kg ERP and 0.3 kg AgCOTE 18/6/8/2+ME, is the most cost-effective fertiliser application while at the same time improving vegetative growth and increasing nutrient uptake efficiency for the planting of first-year oil palm. The insignificant differences in terms of palm height and girth diameter suggest the addition of Polyhalite into a conventional manuring program is comparably effective even at reduced dosage. Additionally, the incorporation of Polyhalite into planting holes during transplanting, together with rock phosphate and CRF at 0.3 kg/tree, respectively, can save up to 40% of the fertiliser cost and represents a dual-strategy for nutrient longevity. A primary limitation of this study was the small, localised sample size causing the findings may not be widely generalisable to other demographics. While this study provided preliminary insights, future studies should include a broader, national sample to validate these findings. Besides that, there were no significant growth differences observed in year one, but strong uptake signals are present, indicating that the lag effects are possible and longer-term monitoring of the study is recommended. Further research should focus on optimising application rates and integrating Polyhalite into different soil and cropping systems to maximise its agronomic benefits. The study also focuses on agronomic management for first-year oil palm; the empirical assessment on microplastic risk would require another environmental study. Besides that, future studies can further evaluate the impact of calcium from Polyhalite on the root growth, as well as the nutrient leaching when applying Polyhalite.

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Valorisation of Kopyor Coconut Waste for Fatty Acid Production by *Yarrowia lipolytica*

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ABSTRACT

Yarrowia lipolytica yeast is a versatile platform that can be used to produce high-value fatty acids from various feedstocks, including the underutilised kopyor coconut. This study aims to explore the differences in valuable lipid-based metabolites from different types of kopyor coconut through fermentation by *Y. lipolytica*. The experiment in this study used three levels of coconut oil concentration (2.5%, 5%, and 7.5%) derived from kopyor coconut waste (var. Genjah and Dalam), which was further processed to obtain oil, and variations in fermentation time (0, 24, 48, 72, and 96 h). The concentration of kopyor coconut oil added to the fermentation medium influenced the growth of *Y. lipolytica*; higher concentrations led to greater cell biomass. After 96 hours of incubation, fatty acids are detected in the cell extract. Substrate fermentation influences the fatty acid profile of *Y. lipolytica*. Yeast Nitrogen Base (YNB) media without coconut oil addition produced alkanes as the majority compound. Meanwhile, YNB with coconut oil addition from var. Genjah produced the usual various fatty acid profiles. The addition of coconut oil from var. Dalam to *Y. lipolytica* pellets resulted in the production of unusual fatty acids with complex derivation. These various free fatty acids hold promise for important applications in biodiesel, nutraceuticals, and food flavouring agents.

Keywords: Fatty acid, kopyor coconut, value-added compounds, *Yarrowia lipolytica*

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INTRODUCTION

Kopyor coconut is a rare and highly prized variety of coconut, characterised by its soft, jelly-like endosperm, which sets it apart from the more commonly consumed mature coconut. Kopyor coconut is a natural genetic mutation of the coconut plant (*Cocos nucifera* L.), which has soft-textured fruit flesh that does not stick to the shell. According to BPS data (2022), the area of kopyor plantations in Central Java has continued to increase from 2016 to 2021, reaching 477.46 hectares with a total production of 1.120.357 tons. Furthermore, the Oil Palm Research Centre developed kopyor coconut seedlings with tissue culture technology that is capable of producing 10-15 thousand seedlings per year.

Kopyor coconut has a unique nutritional profile compared to regular coconuts. While the exact values can vary based on factors like maturity and growing conditions, kopyor coconut generally contains 90% moisture content and a substantial amount of lipids, primarily in the form of medium-chain triglycerides, which are readily digested and absorbed, offering potential health benefits (Dunn et al., 2023). Kopyor coconut contains 30.71% fat, 4.93% protein, and 62.30% carbohydrates (Santoso et al., 1996). Although coconut kopyor is valued for its unique culinary properties, its production generates large amounts of waste, such as oil-rich coconut waste. The unique composition of kopyor coconut, particularly its moisture content and unique lipid profile, makes it a highly sought-after delicacy. However, it also poses challenges in processing and storage due to its perishable nature (Maskromo & Sudarsono, 2013). Kopyor coconut waste can be utilised to create products with high economic value. One approach is through microbial fermentation. This fermentation process can produce compounds with high added value and is environmentally friendly.

According to the Indonesian Oil Palm Research Institute (IOPRI), approximately 20% of kopyor coconuts are produced as waste during processing, demonstrating the potential of this resource for bioconversion (IOPRI internal report, 2020). Research on the utilisation of kopyor coconut waste holds great promise for maximising the utilisation of this valuable resource. Utilising kopyor coconut waste offers the opportunity to convert this underutilised resource into valuable products, such as fatty acids, using microorganisms such as *Y. lipolytica* (Wang et al., 2022). *Y. lipolytica* has been extensively studied as an oil-producing yeast and has been identified as safe (GRASS). This yeast appears to be a host for the production of many valuable compounds (Cao et al., 2022). *Y. lipolytica* can efficiently utilize a variety of carbon sources, including various oils and fatty substrates, to produce lipids and other valuable metabolites (Friedlander et al., 2016).

Many studies have shown the potential of *Y. lipolytica* to produce a wide range of fatty acids from various raw materials as a method for creating new and renewable energy sources (Liu et al., 2021; Lu et al., 2021; Pereira et al., 2022). Therefore, in this study, we aimed to investigate the potential of *Y. lipolytica* for the valorisation of kopyor coconut waste through the production of high-value fatty acids.

MATERIALS AND METHODS

Materials

The research uses *Y. lipolytica* JCM 2320, Yeast Extract Peptone Dextrose (Himedia), Yeast Nitrogen Base (Himedia), aluminium foil, aquadest, KH_2PO_4 (Merck), kopyor coconut waste from the Indonesian Oil Palm Research Institute. The kopyor coconut waste was further processed to extract kopyor coconut oil using the traditional wet method, followed by a filtration step to remove residual solids (Hutauruk, 2017).

Extraction of Kopyor Coconut Oil

The kopyor coconut oil used in this study was obtained through a traditional extraction method described by Simuang et al. (2004). The initial step is to produce the size of the coconut flesh by grating it. The grated coconut flesh is mixed with water to produce coconut milk. The coconut milk is heated at 70-90 °C to reduce the water content, leaving a thick pulp. The resulting oil is separated from the pulp by filtration. To optimise the filtration process, the pulp must be pressed to extract the oil. This process produces the oil used in the experiment.

Substrate Characterisation

Analysis of the physicochemical properties of kopyor coconut oil includes density measurement using an Anton Paar DMA 4500M densitometer in accordance with ASTM D4052, viscosity measurement using a Brookfield DV-III ultra-viscometer in accordance with ASTM D7042, carbon content analysis using spectrophotometry (Rayment & Higginson, 1992), and nitrogen content analysis using the Kjeldahl method (ACIAR, 1990).

Preparation for *Yarrowia lipolytica*

The growth medium for *Y. lipolytica* was YPD (Yeast Extract Peptone Dextrose) agar, which was prepared with 20 g L^{-1} yeast extract, 10 g L^{-1} peptone, 20 g L^{-1} glucose, and 15 g L^{-1} agar (Kim et al., 2013). The growth medium for *Y. lipolytica* was YPD (Yeast Extract Peptone Dextrose) agar, prepared with 20 g L^{-1} yeast extract, 10 g L^{-1} peptone, 20 g L^{-1} glucose, and 15 g L^{-1} agar (Kim et al., 2013). Ingredients were dissolved in distilled water, sterilised at 121 °C and 1 atm for 15 minutes, and poured into sterile petri dishes (15 mL each) to solidify. Liquid YPD medium was prepared with the same composition, omitting agar, and distributed into 100 mL Erlenmeyer flasks (10 mL each) post-sterilisation.

Y. lipolytica was pre-cultured by inoculating a yeast loop in 10 mL YPD in a 100 mL flask, incubating at 30 °C and 250 rpm for 96 hours. The culture was streaked on YPD agar, incubated at 30 °C for 24 hours, and used as stock (Marella et al., 2019).

Preparation of Fermentation Media

The fermentation media for *Y. lipolytica* were prepared following the method of Marella et al., (2019), with modifications for kopyor coconut oil. Kopyor coconut oil at concentrations of 2.5%, 5%, and 7.5% was mixed into the media, and the pH was adjusted to 5.5-6 (Yu et al., 2014). The media composition included 6.7 g L^{-1} YNB (Yeast Nitrogen Base without Amino Acids), 12 g L^{-1} KH_2PO_4, and 10 g L^{-1} glucose. Media preparation was conducted for two kopyor coconut oil varieties, *Dalam* and *Genjah*. Each fermentation medium was prepared at a volume of 150 mL.

Inoculation and Fermentation in Treatment Media

Y. lipolytica was inoculated into a 100 mL Erlenmeyer flask containing 10 mL YPD medium and incubated at 30 °C, 250 rpm for 24 hours in a shaker incubator (Yu et al., 2014). For fermentation, 10 mL of the inoculated YPD medium was added to the prepared fermentation media. The flask was sealed and incubated at 30 °C, 250 rpm for 120 hours (Marella et al., 2019).

Sample Preparation for Analysis

Samples of 5 mL were collected from each fermentation media treatment. For cell density analysis, the samples were directly placed in centrifuge tubes. For wet cell weight, microscopic, and flavour compound analysis, 5 mL samples were centrifuged at 10.000 rpm at 4 °C for 10 minutes to isolate the pellet. The pellet and supernatant were separated using a micropipette, and the pellet was stored at -20 °C for further analysis (Marella et al., 2019).

Cell Density Determination

Cell density was determined by diluting 5 mL of the sample 30 times, transferring the diluted sample into a cuvette, and measuring the absorbance at 600 nm using a UV-Vis spectrophotometer (Celinska, 2013). OD_{600} values were subsequently converted into biomass concentrations (g L^{-1}) using a published conversion factor for *Yarrowia lipolytica* CBS7504 (1 OD_{600} = 0.34 g L^{-1}) (Walker et al., 2021).

Wet Cell Weight Measurement

The wet cell weight was measured by weighing an empty centrifuge tube and then weighing the tube containing the cell pellet using an analytical balance. Before weighing, the pellets were washed with deionised water to remove residual medium components. Afterwards, the pellets were centrifuged again to obtain clean pellets. The difference in weight was recorded as the wet cell weight (Puspitasari & Habibah, 2021).

Microscopic Analysis

Microscopic analysis was conducted using pellets from the fermentation samples, which were washed twice with 2 mL of 10 mM KH_2PO_4 solution. The samples were then centrifuged at 10,000 rpm, 4 °C, for 10 minutes. A final wash with 3 mL distilled water was followed by another centrifugation. The pellet was smeared onto a glass slide, mixed with distilled water, and covered with a cover glass. The morphology of *Y. lipolytica* was observed under a microscope at 100× magnification (Marella et al., 2019).

Fatty Acid Compound Analysis

The fermented pellets were first lysed with 1 mL of distilled water using a sonicator at a speed of 30 for 5 minutes. Then, 3 mL of n-Hexane solvent was added, and the sample was lysed again under the same conditions. Following this, the sample was centrifuged at 10.000 rpm, 4 °C, for 10 minutes. The top layer of the centrifugation was collected (1 mL), filtered using a 0.45 µm syringe filter, and placed in a vial for gas chromatography-mass spectrometry (GC-MS) analysis.

Free fatty acid (FFA) compound analysis was conducted using gas chromatography-mass spectrometry (GC-MS). A 1 µL sample was injected into the GC injector, set to an initial temperature of 250 °C and operated in splitless mode for 2 minutes, then switched to split mode with a 50:1 ratio. Helium gas was used as carrier gas, with a constant flow rate of 1 mL/min. The gas chromatography column temperature was set at 50 °C for 1 minute, then increased at a rate of 10 °C per minute until it reached 210 °C, where it was maintained for 10 minutes. The temperature was then increased to 230 °C at 10 °C per minute and maintained for 5 minutes (Marella et al., 2019).

The compound yield was estimated by multiplying the relative area percentage of each compound by the total extract mass obtained from each sample, followed by normalisation to the corresponding wet cell weight (WCW) (Alseekh et al. 2021). The results were expressed as compound yield (g compound g^{-1} wet cell weight), as shown as in Equation 1.

$$Y_i = \frac{A_i}{100 \times m_{\mathrm{WCW}}} \qquad [1]$$

where:

- Y_i = yield of compound *i* (g compound g^{-1} wet cell weight)
- A_i = relative GC-MS peak area of compound *i* (%)
- m_{WCW} = wet cell weight (g)

Statistical Analysis

This study examined three factors: kopyor coconut varieties, media substitution percentage, and fermentation time. This study used *Genjah* and *Dalam* kopyor coconut varieties as media types, with the addition of kopyor coconut oil at three levels: 2.5%, 5%, and 7.5% (v/v) of the total growth medium. Fermentation time was observed at 24, 48, 72, and 96 hours. Each combination of coconut varieties, media substitution percentage, and fermentation time was repeated three times. All data were analysed using one-way analysis of variance (ANOVA) with a 95% confidence level. If a significant effect was found (P value <0.05), Duncan's test was used to assess the significance of the difference between treatments. Statistical analysis was performed using the SPSS Statistics 26.

RESULTS AND DISCUSSION

Physicochemical Properties of Kopyor Coconut Oil

The physicochemical properties of kopyor coconut oil can be seen in Table 1, which include viscosity, density, carbon, and nitrogen. The potential of kopyor coconut oil, particularly as a substrate for *Y. lipolytica*, can be seen from the listed parameters. This is important to note because viscosity and density can affect the dispersion of substrate availability in aqueous media, while nitrogen and carbon are nutrient sources for cell growth and metabolic activity (Singh et al. 2017).

Table 1
Physicochemical properties of kopyor coconut oil

	Density (kg/m^3)	Viscosity (mm^2/s)	Carbon (%)	Nitrogen (%)	Lipid (%)
Kopyor Coconut Oil	906.90	29.06	232.02	0.10	99.91

Effect of Substrate Concentration and Fermentation Time on *Yarrowia lipolytica* Cell Density on Kopyor Coconut Oil Substrate (Var. Genjah and Dalam)

The growth of *Y. lipolytica* on both kopyor coconut oil substrates (var. Genjah and var. Dalam) showed a comparable trend (Figure 2). The results from both substrates showed a decrease in cell density during the first 24 hours. This is closely related to the yeast adaptation phase to the media conditions. These results are in line with the Ginovart et al. (2011) study, which reported that yeast cells are metabolically active during the lag phase. The absorbance values at 48 hours from high to low concentrations in var. Genjah were respectively: 7.5% (0.095 ± 0.03), 5% (0.227 ± 0.07) and 2.5% (0.289 ± 0.012). Meanwhile, the absorbance values at 48 hours from high to low concentrations in var. Genjah at 7.5% concentration (0.095 ± 0.03), followed by 5% (0.227 ± 0.07) and 2.5% (0.289 ± 0.012). Similarly, for var. Dalam, the highest absorbance value was observed at 7.5%

(0.363 ± 0.01), with 5% (0.245 ± 0.006) and 2.5% (0.145 ± 0.014) following suit. Based on these results, it can be seen that substrate concentrations generally encourage more cell growth in the exponential phase. The increased cell density at high concentrations (7.5% and 5%) indicates that yeast is able to utilise fatty acids such as caprylic and myristic acids as carbon sources (Rigoun et al. 2017). These fatty acids have the advantage of being efficiently degraded, providing energy, and serving as building blocks for biomass production. The higher cell density than the control medium (YNB) also supports this conclusion.

At a concentration of 2.5%, cell density was lower than the control medium. For example, in var. Dalam, cell density peaked at 72 hours with an absorbance value of 0.145 ± 0.014, this indicates that the nutrients contained in the medium are starting to decrease and byproducts are also starting to form in large quantities. This occurred in both substrates with a significant decrease at low concentrations. Although the growth pattern was similar in both varieties, var. Dalam showed a higher cell density at all concentrations. The comparison of absorbance values in the two varieties with the highest concentration value was 0.363 ± 0.01 (var. Dalam) and 0.289 ± 0.012 (var. Genjah). This difference is due to the higher fatty acid content in var. Dalam. This is able to encourage better growth and biomass production (Soong et al., 2019). The following is a graph of *Y. lipolytica* cell density in fermentation media with kopyor coconut oil substrate (var. Genjah and Dalam).

The conclusion of this study is that *Y. lipolytica* can grow better on kopyor coconut oil substrate var. Dalam with high concentrations (7.5% and 5%).

To complement the growth curve data shown in Figure 1, the OD_{600} values obtained in this study were converted to estimated cell mass (g/L). The conversion was performed using a published factor, where 1 OD_{600} = 0.34 g/L for *Y. lipoytica* CBS7504 (Walker et al., 2021). Table 2 presents the obtained OD_{600} values along with the corresponding estimated cell mass at the observed peak growth for each substrate concentration. While this conversion provides only estimates, as actual values may vary depending on strain and culture conditions, it still provides a useful context for interpreting optical density-based growth patterns in relation to biomass calculation.

Table 2
OD_{600} values and corresponding estimated cell mass (g/L) of Yarrowia lipolytica *at peak growth under different substrate concentrations*

Yarrowia lipolytica	**OD_{600}**	**Cell Mass (g/L)**
Genjah and Dalam 0%	0.235±0.011	0.080±0.004
Genjah 2.5%	0.141±0.008	0.048±0.003
Genjah 5%	0.227±0.003	0.077±0.001
Genjah 7.5%	0.289±0.012	0.098±0.004
Dalam 2.5%	0.145±0.014	0.049±0.005
Dalam 5%	0.245±0.006	0.083±0.002
Dalam 7.5%	0.363±0.010	0.123±0.003

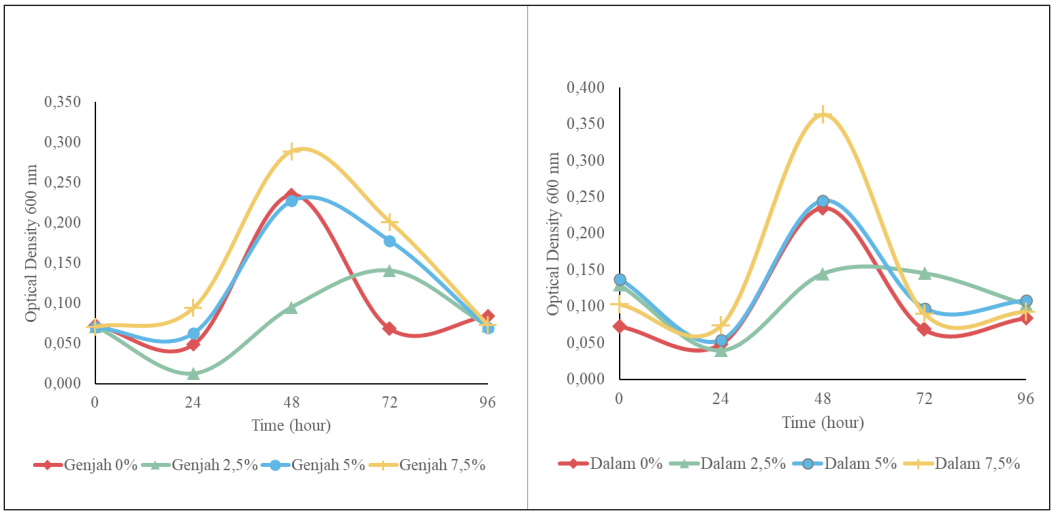

Figure 1. Cell density of *Yarrowia lipolytica* in fermentation medium + coconut oil kopyor var. Genjah and var. Dalam

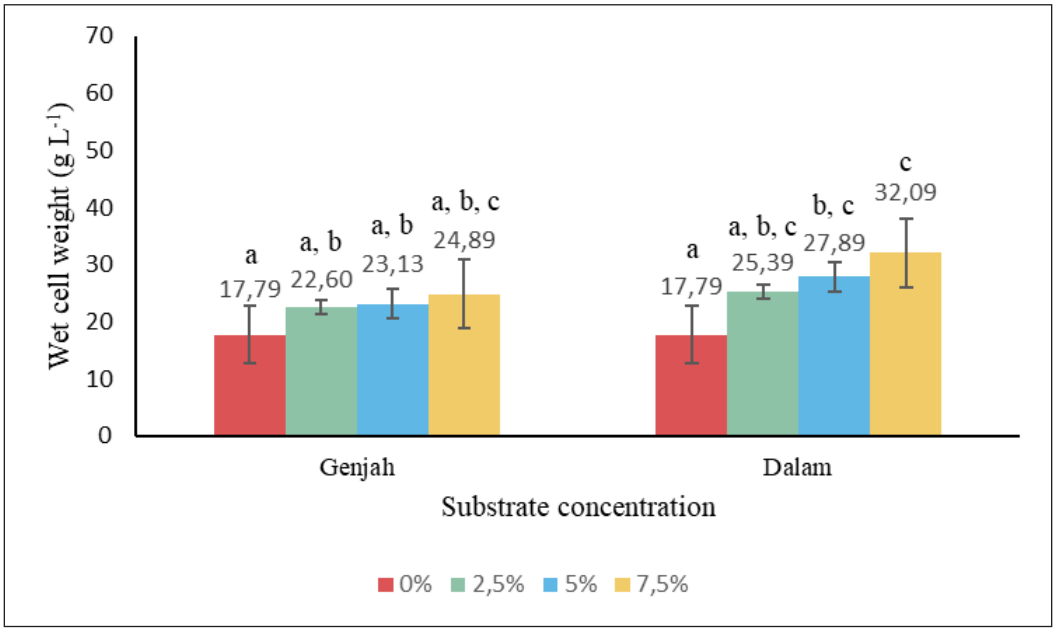

Figure 2. Wet weight curve of *Yarrowia lipolytica* cells on various media with a fermentation time of 72 hours

Note. Means in the same column followed by the same letter are not significantly different according to Duncan's multiple range test at $\alpha = 0.05$

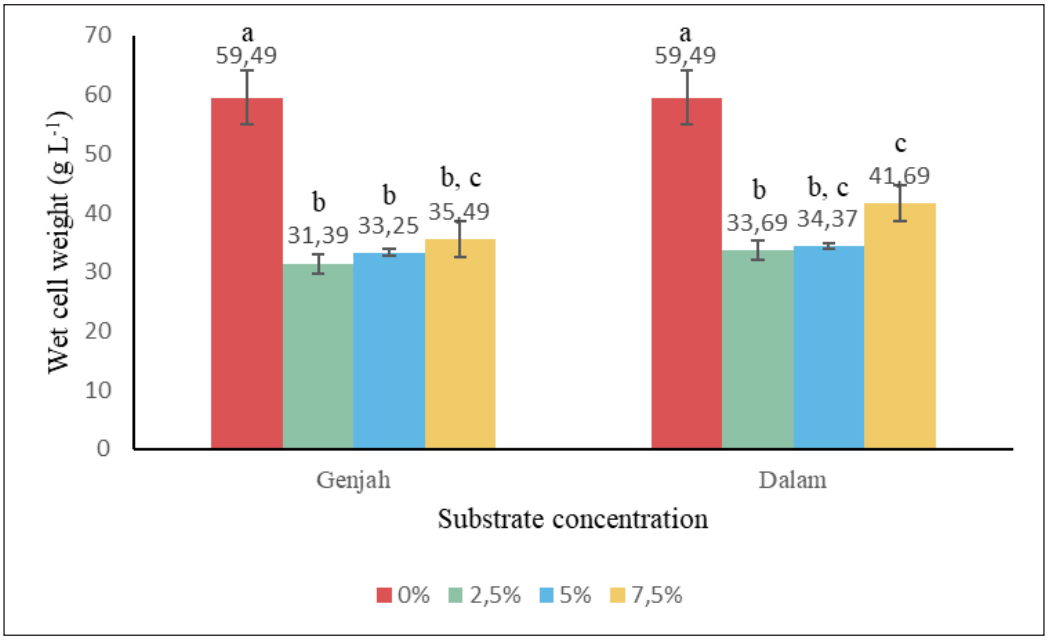

Figure 3. Wet weight curve of *Yarrowia lipolytica* cells on various media with a fermentation time of 96 hours

Note. Means in the same column followed by the same letter are not significantly different according to Duncan's multiple range test at $\alpha = 0.05$

Wet Cell Weight of *Yarrowia lipolytica* Cells in Kopyor Coconut Oil Substrate

Wet cell weight provides a direct estimate of biomass concentration and can be used to obtain information about the growth of microorganisms such as *Y. lipolytica* (Choi et al., 2013). In this study, the wet weight of *Y. lipolytica* was measured using an analytical balance to observe biomass fluctuations during the fermentation process.

Fermentation of *Y. lipolytica* in kopyor coconut oil substitute medium showed an increase in wet weight from 72 to 96 hours (Figure 2 and Figure 3). This increase indicates cell growth and can be used as an important parameter to evaluate the performance of *Y. lipolytica* during fermentation.

However, wet cell weight does not always correlate directly with cell density. Wet weight is influenced by the rate of cell division (Bryan et al., 2009) as well as variations in the mass of individual cells. Variability in individual cell weight can lead to conditions where samples have high cell density but relatively low cell mass, or vice versa (Zhao et al., 2014).

ANOVA results indicated that fermentation on coconut oil substitution media produced significantly higher wet weight values compared to the control (YNB). Variations in oil type (var. Genjah and var. Dalam) and concentration significantly influenced wet weight at

72 hours ($p < 0.05$). Duncan's test revealed that var. Dalam at 5% and 7.5% concentrations yielded significantly higher wet weight increases.

At 96 hours, significant differences ($p<0.05$) were observed between treatments. Duncan's test identified var. Dalam at 7.5% concentration as the best treatment, with the highest wet weight among substituted media. However, compared to the control, this value remained lower. This indicates that var. Dalam media at 7.5% concentration has substantial potential, although the control remains more effective for wet weight production at this time point.

On average, the wet weight of *Y. lipolytica* cells increased by 31% from 72 to 96 hours in the substitution media. Across all concentrations, var. Dalam produced a higher wet cell weight compared to the var. Genjah. This difference is related to factors that influence cell division and growth, such as nutrient availability, temperature, pH, and fermentation medium composition (Talavera et al., 2024). However, an increase in wet weight does not always correlate with an increase in cell density. Physiological differences between cells can affect individual mass and cell density within the resulting biomass (Zhao et al., 2014). In many cases, higher wet weight reflects more intense metabolic activity, which may be related to increased production of fatty acid compounds (Braga et al., 2022).

Morphological Characteristics

Y. lipolytica is a dimorphic yeast that is able to grow in two morphological forms, namely yeast-like cells and short mycelium cells (Ruiz-Herrera & Sentandrey, 2002). In control medium (YNB), *Y. lipolytica* showed both yeast-like and short mycelial forms, with round to oval cells, some forming chains, and having a smooth surface. Morales-Vargas et al. (2012) stated that under natural conditions, *Y. lipolytica* generally grows as a mixture of yeast and short mycelial cells. However, under more controlled culture conditions, this microorganism can also grow as yeast-like cells or branching hyphae.

Microscopic observations of *Y. lipolytica* grown in kopyor coconut oil substitution media showed various morphological forms at different oil concentrations in Figure 4. At a 2.5% coconut oil substitution (var. Genjah), the cells displayed a mycelial form with pseudohyphae, forming elongated chains. This is consistent with Zinajarde et al. (2008), who observed that mycelial formation occurs when certain crude oils, such as coconut oil, are used as carbon sources. At a 5% coconut oil substitution, the cells adopted a yeast-like shape with round and uniform characteristics. At 7.5%, the cells transitioned back to mycelial form with pseudohyphae, forming chains, a finding also reported by Papanikolaou et al. (2007), who noted that the yeast-to-mycelium transition is common when fats containing lauric, myristic, palmitic, oleic, and stearic acids are used as carbon sources.

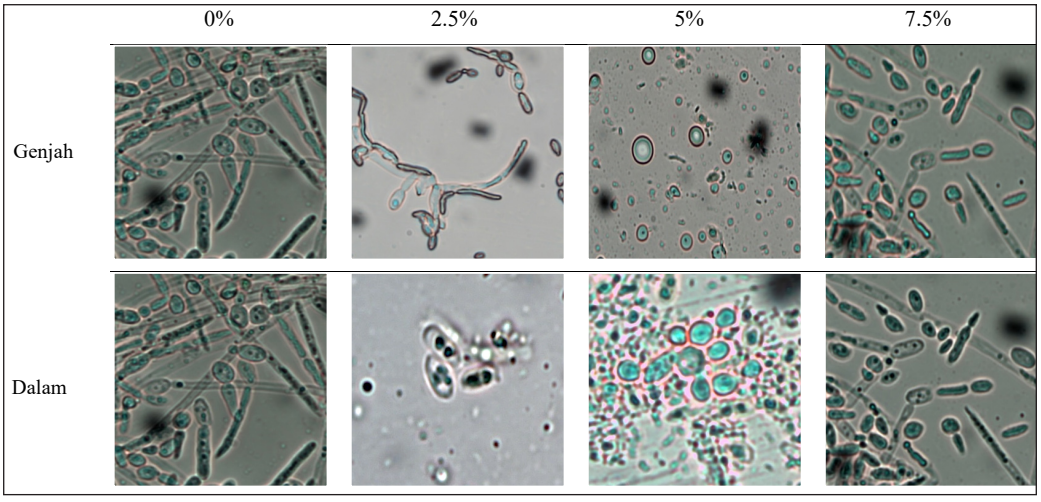

Figure 4. Microscopic observation of *Y. lipolytica* with a magnification of 100×

For the coconut oil, kopyor substitution with Var. Dalam, at 2.5%, the cells retained a yeast-like shape, with round to oval cells and uniform size. At 5%, the cells were yeast-like, with some cells attached in pairs. At 7.5%, the cells showed both yeast and mycelial forms, with pseudohyphae forming long chains. Similar to var. Genjah, these changes can be attributed to the types of carbon sources, such as glucose and fatty acids, and nitrogen sources like ammonium, as well as factors like low oxygen availability in the medium (Braga & Belo, 2022; Zinajarde et al., 2008).

In conclusion, *Y. lipolytica* can adapt its morphology in response to different fermentation conditions, particularly the composition of the media. However, as noted by Małajowicz and Kozłowska (2021), the shape of the yeast does not directly correlate with the production of bioflavour compounds, such as lactones, which are influenced by metabolic pathways, enzymatic activities, and environmental factors.

Profiling FFA Compound

In this study, *Y. lipolytica* was fermented using minimal media of YNB added with coconut oil Kopyor. Hydrophobic substrates like coconut oil can activate *Y. lipolytica* to produce extracellular lipase, and then the oil can be hydrolysed into fatty acids and glycerol. The incorporation of these fatty acids into the cytosol led to the activation of FAA1 (fatty acyl-CoA synthetase) in order to produce acyl-CoA (Rakicka et al., 2015). Acyl-CoA is a building block for fatty acyl-CoA to synthesise some compounds, such as essential fatty acids (EFA), alkanes, fatty alcohols, fatty acid ethyl esters (FAAE), and free fatty acids (FFA) (Liu et al., 2021). Meanwhile, hydrophilic substrates such as glucose and glycerol can be converted into fatty acids via the *de novo* pathway (Ledesma-Amaro & Nicaud, 2016).

To identify the fatty acid compounds produced during *Y. lipolytica* fermentation, we investigated the fatty acid profile from pellets using Gas chromatography-mass spectrometry (GC-MS) methods. The sample preparation focussed on the pellet fraction, as pellets are known to accumulate fatty acid compounds from kopyor fermentation. This is due to the production and accumulation of intracellular fatty acid compounds within yeast cells (Pensupa et al., 2023). Furthermore, Colacicco et al. (2022) reported that cell pellets retain cellular activity essential for fatty acid production, making cell biomass a rich source of these compounds. The use of pellets as samples can also improve the efficiency of fatty acid extraction. This is because supernatants typically contain various dissolved culture media components that do not always represent the total fatty acid production by the microbes (Naveira-Pazos et al. 2023).

The GC-MS chromatogram indicates the presence of various fatty acid compounds, with the identified compounds presented in Table 3. In the control medium, several dominant compounds detected included octadecane, hexadecane, and heneicosane. These hydrocarbons indicate active lipid biosynthesis by *Y. lipolytica*. Campos-Góngora et al. (2018) reported that saturated hydrocarbons can be formed as byproducts of the lipid degradation process. Furthermore, the presence of compounds such as octadecane and hexadecane may also indicate significant β-oxidation activity under the applied fermentation conditions (Vasiliadou et al., 2018).

In the fermentation medium substituted with kopyor coconut oil, various concentrations produced several dominant compounds such as lauric acid, stearic acid, palmitic acid, myristic acid, and oleic acid. These compounds are important components in lipid metabolism and highlight the potential of *Y. lipolytica* in producing valuable lipids for industrial applications. The presence of lauric, stearic, and oleic acids identified in this study is also aligned with the previous reports that certain fermentation conditions can enhance fatty acid production in *Y. lipolytica* (Wang et al., 2022). Palmitic and myristic acids, key products in lipid biosynthesis, underscore the influence of fermentation conditions on the metabolic pathways of *Y. lipolytica* (Soong et al., 2019).

Genjah 2.5% has the highest dodecanoic acid content among other fatty acids, where the lauric fraction has been reported to be used as a raw material source for renewable energy (Bukhari et al., 2022). The fatty acid content in *Y. lipolytica* can be utilised as an alternative energy source in certain modified engines. Liu et al., (2021)

Genjah 2.5% has the highest dodecanoic fatty acid content among the other fatty acids, where the laurate fraction is reported to be able to be used as a source of raw material for renewable energy (Bukhari et al., 2022). The fatty acid content within *Y. lipolytica* can be utilised as a source of alternative energy in certain modified engines. Liu et al. (2021) discussed the secretion of fatty acid esters by an engineered *Y. lipolytica* strain. This approach eliminates the need for transesterification, simplifying the process and potentially reducing costs.

Table 3
Compounds identified from the fermentation of Y. lipolytica *in various media substrates*

Sample	Retention Time (minutes)	Area (%)	Compound	Yield (g compound g^{-1} wet cell weight)
Control (YNB)	16.576	17.10	Octadecane ($C_{18}H_{38}$)	0.1710
	14.361	13.97	Hexadecane (C_6H_{34})	0.1397
	18.914	9.77	Heneicosane ($C_{21}H_{44}$)	0.0977
	32.022	3.86	Fumaric acid ($C_4H_4O_4$)	0.0386
	32.244	2.95	Glycerol tricaprylate ($C_{27}H_{50}O_6$)	0.0295
	16.348	0.78	Carbonic acid (H_2CO_3)	0.0078
Genjah 2.5%	14.188	41.55	Dodecanoic acid ($C_{12}H_{24}O_2$)	0.4155
	16.320	20.44	Octadecanoic acid ($C_{18}H_{36}O_2$)	0.2044
	18.534	12.48	n-Hexadecanoic acid ($C_{16}H_{32}O_2$)	0.1248
	21.527	10.23	Oleic acid ($C_{18}H_{34}O_2$)	0.1023
	25.774	0.05	Propanoic acid ($C_3H_6O_2$)	0.0005
	13.417	0.05	2,4-Di-tert-butylphenol ($C_{14}H_{22}O$)	0.0005
Genjah 5%	14.074	34.42	Dodecanoic acid ($C_{12}H_{24}O_2$)	0.3442
	16.259	19.92	Tetradecanoic acid ($C_{14}H_{28}O_2$)	0.1992
	18.486	14.64	n-Hexadecanoic acid ($C_{16}H_{32}O_2$)	0.1464
	21.475	12.55	Oleic acid ($C_{18}H_{34}O_2$)	0.1255
	13.413	0.29	2,4-Di-tert-butylphenol ($C_{14}H_{22}O$)	0.0029
Genjah 7.5%	14.077	38.62	Dodecanoic acid ($C_{12}H_{24}O_2$)	0.3862
	16.259	18.99	Tetradecanoic acid ($C_{14}H_{28}O_2$)	0.1899
	14.485	10.81	n-Hexadecanoic acid ($C_{16}H_{32}O_2$)	0.1081
Dalam 2.5%	14.023	32.14	Dodecanoic acid ($C_{12}H_{24}O_2$)	0.3214
	16.229	15.49	Tetradecanoic acid ($C_{14}H_{28}O_2$)	0.1549
	18.468	14.60	n-Hexadecanoic acid ($C_{16}H_{32}O_2$)	0.1460
	13.406	0.93	2,4-Di-tert-butylphenol ($C_{14}H_{22}O$)	0.0093
Dalam 5%	14.025	29.28	Dodecanoic acid ($C_{12}H_{24}O_2$)	0.2928
	16.278	17.65	i-Propyl 12-methyl-tridecanoate ($C_{17}H_{34}O_2$)	0.1765
	18.514	15.54	l-(+)-Ascorbic acid 2,6-dihexadecanoate ($C_{38}H_{68}O_8$)	0.1554
	13.420	0.09	2,4-Di-tert-butylphenol ($C_{14}H_{22}O$)	0.0009
Dalam 7.5%	14.225	21.27	1,2,4-Trioxolane-2-octanoic acid, 5-octyl-, methyl ester ($C_{19}H_{36}O_5$)	0.2127
	21.561	12.59	6-Octadecenoic acid ($C_{18}H_{34}O_2$)	0.1259
	9.085	12.14	5-Butyl-1,3-oxathiolan-2-one	0.1214
	16.242	12.06	i-Propyl 12-methyl-tridecanoate ($C_{17}H_{34}O_2$)	0.1206

Table 4
Identified FFA compounds and their applications as fine chemicals in the food industry

Medium	Compounds	Applications
Control (YNB)	Octadecane Hexadecane Heneicosane	Production of lubricants, waxes and food packaging materials (Chayabutra & Ju, 2000)
	Fumaric acid	Corn and wheat tortilla flavour ingredients, acidity regulators, and preservatives (Ilica et al., 2018)
	Glycerol tricaprylate	Improved b-pinene flavour release characteristics and stabilisers (Koupantsis et al., 2016)
	Carbonic acid	Provides a carbonation effect on carbonated drinks, alcohol, and kombucha (Pelchat et al., 2014)
	n-Hexadecanoic acid	Affects texture and improves butter quality (Music et al., 2022).
	Oleic acid	Increases the release of aromatic aromas from the Maillard reaction (Benet et al., 2015)
Kopyor coconut oil	Propanoic acid	Gives a sweet taste and nutty aroma to cheese, as a preservative (Bücher et al., 2021)
	2,4-Di-tert-butylphenol	Natural antioxidant (Varsha et al., 2015)
	Tetradecanoic acid	It produces a waxy taste in the mouth that can be enjoyed when combined with other ingredients (Burdock and Carabin 2007)
	l-(+)-Ascorbic acid 2,6-dihexadecanoate	Vitamin C has antioxidant and antimicrobial properties (Mumtaz et al. 2023)

In addition to producing fatty acid components that have the potential to be used as renewable energy feedstock, the utilisation of kopyor coconut waste through microbial fermentation also produces various value-added chemicals with potential for food applications (Table 4). *Y. lipolytica* can be a microbial cell factory for producing various fatty acid-based bioproducts, including nutraceuticals and biochemicals (Liu et al., 2021).

These findings indicate that the media composition and fermentation conditions are capable of directing the metabolism of *Y. lipolytica* toward the production of lipids and their derivatives. The presence of hydrocarbons such as octadecane and hexadecane reflects lipid degradation activity during the fermentation process. Meanwhile, the identification of fatty acids such as lauric, stearic, palmitic, myristic, and oleic acids demonstrates the yeast's ability to synthesise various lipid derivatives with commercial value.

Although *Y. lipolytica* produces relatively lower amounts of fatty acids compared to microalgae, this yeast still offers several advantages. One of the advantages is that the cultivation conditions are simpler. Microalgae require appropriate nutrient inputs, such as carbon, nitrogen, and light, to optimise lipid production (Mou et al., 2023; Udayan et al., 2023). In contrast, *Y. lipolytica* can utilise a broader range of substrates, including

organic waste, for lipid synthesis (Drzymała-Kapinos et al., 2022; Fabiszewska et al., 2021). Moreover, microalgae generally require more complex lipid extraction processes compared to *Y. lipolytica*, as they have thicker and more intricate cell walls. In contrast, *Y. lipolytica* biomass can directly produce lipids in a form that is more readily processable (Lee et al., 2021).

CONCLUSION

Based on the results and discussion, the level of coconut oil substitution in the fermentation media significantly affects the growth of *Y. lipolytica*. A substitution concentration of 7.5% results in a higher increase in *Y. lipolytica* growth compared to the control medium, while a 5% substitution resulted in relatively equivalent growth. Conversely, a 2.5% substitution leads to lower growth. Among the substrates tested, kopyor coconut oil from the Dalam variety consistently produced higher growth than the Genjah variety across all concentrations, as observed through cell density and wet cell weight parameters.

The wet weight of *Y. lipolytica* cells at 72 and 96 hours of fermentation was significantly influenced by the concentration and variation of the media. Substitution with 7.5% kopyor coconut oil (var. Dalam) resulted in the highest wet cell weight compared to the other treatment media, although it did not exceed the control media. Microscopic observations indicated that *Y. lipolytica* is able to adapt well to various fermentation conditions, as evidenced by diverse morphological forms. However, these morphological changes did not correlate with the production of fatty acid compounds.

Overall, utilising coconut waste through microbial fermentation using *Y. lipolytica* is a promising approach to transforming this underutilised resource. This process has the potential to produce high-value fatty acids that can be used as a renewable energy source and other high-value products for the nutraceutical and food industry.

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Phenological Growth Stages of *Artocarpus odoratissimus* using the Extended BBCH Scale and its Applications in Determining Optimal Harvest Maturity

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ABSTRACT

Artocarpus odoratissimus, an indigenous tropical fruit native to Borneo, is highly valued by local communities for its captivating aroma and promising market potential. However, its commercialisation is hindered by limited knowledge of its phenology and inefficient harvesting practices, both of which significantly affect its shelf life. This study aims to elucidate the phenological growth stages and establish maturity indices for *A. odoratissimus* to determine the optimal harvest maturity. The study adopts the extended BBCH scale, employing a three-digit numerical system to define the phenological stages of *A. odoratissimus*, encompassing four principal growth stages: from stage 5 (reproductive part development) to stage 8 (fruit maturation). Fruit maturation progresses through three distinct phases: (i) a 40-day lag phase after anthesis (AA), (ii) a 40-day logarithmic growth phase, and (iii) a declining growth phase lasting 15 to 20 days. Detailed phenological observations indicated that optimal harvesting occurs at ~day 90 AA, when the fruits can be effectively stored, whereas harvesting at day 95 yields fruits suitable for direct consumption. These findings underscore the utility of the BBCH scale as a valuable tool for crop management, particularly for farmers. By providing a clear framework for understanding growth stages, it enables precise harvest timing, reduces fruit wastage, enhances fruit quality, and improves the marketability of *A. odoratissimus* produce.

Keywords: Artocarpus odoratissimus, BBCH scale, climacteric fruit, optimum maturity, phenology, underutilised fruit

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INTRODUCTION

Plant phenology is the study of seasonal changes in plant life cycles, which are highly species-dependent and profoundly influence crop production dynamics (Richardson et al., 2013). Phenological events such as bud emergence, flowering, fruiting, and senescence are closely linked to specific growth stages across plant species and have critical implications for agricultural practices (Chuine & Régnière, 2017). The BBCH (*Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie*) numerical scale is widely used to delineate phenological stages in plants (Meier et al., 2009). It provides a standardised coding system for identifying and describing phenologically equivalent growth stages in both monocotyledonous and dicotyledonous plants. The scale employs a two-digit code, where the primary digits (0-9) represent the main growth stage, and the secondary digits (0-9) represent the sub-stage (Lancashire et al., 1991). The BBCH scale provides a detailed framework that enables farmers and researchers to synchronise agricultural practices with plant development, thereby improving decisions regarding planting, fertilisation, pest control, and harvesting (Meier et al., 2009). This comprehensive scale has been expanded to broad spectrum of plant species, such as dragon fruits (Chu & Chang, 2022; Kishore, 2016), java plum (Singh et al., 2021), hazelnuts (Taghavi et al., 2022), rambutan (Muhamed & Kurien, 2018), longan (Pham et al., 2015), and jackfruits (Kishore, 2018).

Artocarpus odoratissimus Blanco is an indigenous, climacteric fruit native to Southeast Asia, particularly Borneo, including Sarawak, Sabah, and Brunei. It has also been introduced to other countries, such as the Philippines, Australia, and Brazil (Bakar & Bakar, 2018). This fruit belongs to the Moraceae family, which comprises ~60 genera and over 1,400 species (Jagtap & Bapat, 2010). The most popular species from this genus include *Artocarpus heterophyllus* (Jackfruit), *Artocarpus integer* (Cempedak), and *Artocarpus altilis* (breadfruit). *Artocarpus odoratissimus,* commonly known as terap, tarap, or marang, is characterised by its spiky green exterior, which turns brownish-yellow when ripe and emits an aromatic fragrance. The juicy flesh has gained popularity in local markets and is among the most widely consumed fruits in Borneo, as reported by Karson et al. (2020). This plant holds significant economic and nutritional value, making it a highly advantageous multifunctional crop for both fruit and timber production (Zerega et al., 2005). Furthermore, *A. odoratissimus* is a rich source of essential minerals that contribute to managing conditions such as anxiety, stroke, blood pressure, and cardiovascular and renal health (Martinez & Perez, 2016).

However, *A. odoratissimus* is often classified as an underutilised fruit, primarily due to its highly perishable nature and rapid deterioration (Reddy et al., 2010). The key factors contributing to this issue include improper harvesting periods and practices. Delayed harvesting often results in fruits being picked when they are overly mature, significantly reducing their shelf life (Bakar & Bakar, 2018; Mondal et al., 2013).

The lack of information on the phenological events and standardised harvesting protocols leads to variations in maturity levels, causing the fruits to degrade more rapidly.

Understanding the phenological development of crops is crucial for optimising agricultural practices, particularly to ensure harvesting at the ideal time. This approach can help extend shelf life and maximise the value of agricultural produce. The present study aims to elucidate the phenological growth stages and establish maturity indices for *A. odoratissimus*, enabling the determination of optimal harvest maturity. This study seeks to empower growers with precise crop management techniques to ensure optimal harvest timing, preserving fruit quality, and extending shelf life.

MATERIALS AND METHODS

Study Area and Sample Collection

The study was conducted at a fruit orchard in Kampung Pasir Pandak, Kuching, Sarawak (N 4˚3'29.466" and E 113˚50'39.0948"). Sampling locations were marked using a Garmin GPSMAP® 64S to obtain the GPS coordinates. Data on vegetative and reproductive phases at different developmental stages were collected from 10 to 12-year-old plants of five *A. odoratissimus* trees from January 2022 to September 2023. In 2022, the total monthly precipitation ranged from 153.00 mm to 793.60 mm, and temperatures ranged from 25.83 °C to 27.50 °C. In 2023, the total monthly precipitation ranged from 164.80 mm to 817.40 mm, with temperatures ranging from 26.04 °C to 27.72 °C (Figure 1) (Kuching Meteorological Department, 2024).

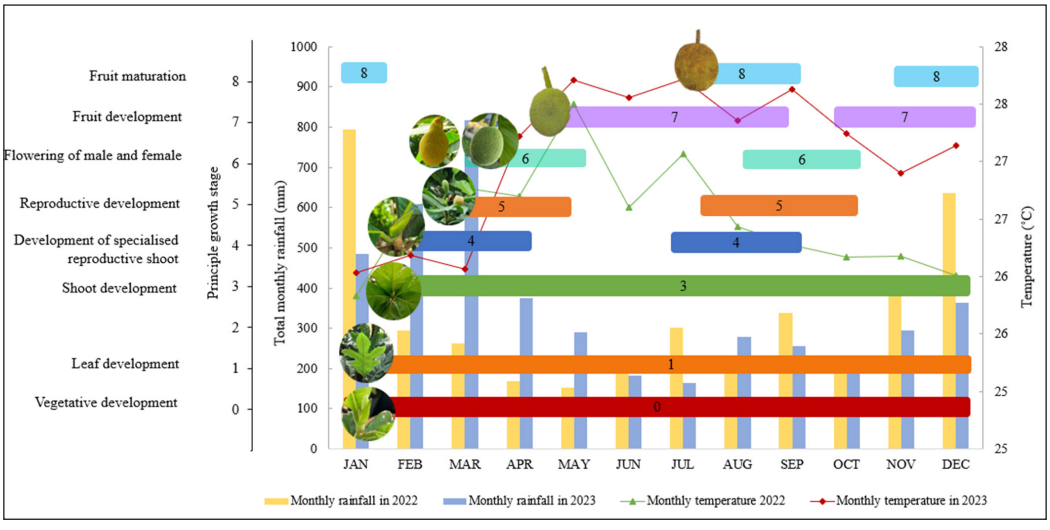

Figure 1. Principal growth stages of *Artocarpus odoratissimus* in relation to total amount of rainfall and temperature from 2022-2023 (Department of Meteorology Kuching, 2024). The bar represents the elapsed time of different growth stages

Phenological Observation

Data on various phenological stages of bud development and reproductive development were recorded at 5-day intervals. Flower buds and fruits were randomly selected across the canopy, with emphasis on mid-canopy positions, and were tagged for phenological observation. Fruit samples were subsequently collected from these tagged structures. The proposed extended BBCH scale for *A. odoratissimus* follows that of jackfruit, which is represented by four principal growth stages, starting with stage 5 (reproductive development), 6 (flowering), 7 (fruit development), and 8 (fruit maturity). Stage Principal growth stages were further divided into 10 secondary stages (0-9) corresponding to intermediate developmental stages linked to specific stages. These stages represent qualitatively different stages within a given principal growth stage. Mesostages (1, 2, 3, …) were used to define the frequency of occurrence of principal growth stages during a crop cycle. For example, code 815 represents the fruit maturation stage, indicating that the fruit is fully developed, matured, and will undergo ripening next. Besides, because *A. odoratissimus* is a monoecious plant wherein male and female inflorescences (spikes) are borne separately, stages 5 and 6 have been described separately for male and female spikes. The phenological growth stages and fruit development were sequentially characterised and photographed by the same observer throughout the study to ensure consistency.

Determination of Fruit Maturity Indices of *Artocarpus odoratissimus*

To study the maturity indexes of *A. odoratissimus*, random sampling was done to collect the fruits (8-10 fruits) at Stage 8 (fruit maturation) with five maturity stages including 811 Beginning of fruit maturation (80-85 days AA), 815 Advanced fruit maturation (90-94 days AA), 817 Fruit fully mature for picking (95-99 days AA), and 819 Fruit over mature (100-105 days AA). The collected fruit samples were immediately cleaned and analysed to determine general properties following standard protocols.

Determination of Fruit Physicochemical Properties

Fruit mass and size were measured using an electronic balance and a measuring tape. The fruit exocarp colour was assessed based on a scale ranging from 0 to 5, representing the progression from dark green to deep brown (0 = dark green, 1 = light green, 2 = greenish yellow, 3 = brownish yellow, 4 = brown, 5 = deep brown), while the flesh scale also ranging from 0 to 5, representing the progression from clear white to fully brown (0 = white, 1 = slightly whitish yellow, 2 = equal white and yellow, 3 = fully yellowish, 4 = slightly brownish, 5 = fully brown).

Additionally, colour coordinates as $L^* a^* b^*$ scale system were recorded from the fruit surface using a Colour Reader CR10. The fruit's firmness was measured using a penetrometer.

Penetrometer readings were converted to newton (N) values. The total soluble solids (TSS) were assessed using a hand-held refractometer at standard temperature (20˚C), and the results were expressed in ˚Brix (method 983.17, AOAC, 2000). The pH of the fruit was determined by using a pH meter (964.24 (AOAC, 2000).

Statistical Analyses

Data for physicochemical properties were triplicated and statistically analysed using SAS 9.4 Windows software (SAS Institute Inc, Cary, USA). Means were compared using single-factor analysis of variance (ANOVA) to compare physicochemical attributes among discrete maturity stages. Post hoc Tukey's test (p<0.05) was performed if the ANOVA result was significant.

RESULTS AND DISCUSSION

Phenological Event of *Artocarpus odoratissimus*

Phenology refers to the annual progression of biological development stages in plant species (Singh et al., 2023). The phenology of *A. odoratissimus* in Sarawak shows a remarkable cycle intertwined with the region's tropical climate. The success of this plant is reflected in its ability to adapt to Sarawak's distinct seasons, as evidenced by abundant fruit production during its peak season. The phenological activity observed in this study is shown in Figure 1. This fruit tree displays a distinct phenological cycle, characterised by continuous vegetative growth throughout the year, as the climatic conditions consistently support year-round development. Major flowering was recorded from March to May and from August to October, coinciding with lower to moderate rainfall in 2022, ranging from 153.00 mm to 338.80 mm and from 218.80 mm to 374.00 mm, respectively. However, in March 2023, an exceptionally high rainfall of 817.40 mm was recorded, which impacted the flowering process. The temperature ranged from 26.17 °C to 27.50 °C in 2022, and from 26.06 °C to 27.7 °C in 2023.

The *A. odoratissimus* fruiting season in Sarawak occurs twice a year, with the major season from June to August, while the minor season is from November to January. From June to August, the region experiences lower to moderate rainfall, ranging from 164.80 mm to 302.80 mm, with temperatures ranging from 26.43 °C to 27.72 °C, in both 2022 and 2023. In contrast, higher rainfall was recorded during the fruiting season from November to January, ranging from 294.40 mm to 793.60 mm, while temperature ranged from 26.01 °C to 27.14 °C in both years. In the Philippines, the fruiting season is from May to July in Luzon and from August to December in Mindanao. The flowering and fruiting in Borneo indicate that the fruit ripens between October and January (Bakar & Bakar, 2018). In Northern Queensland, Australia, the fruit was harvested over two months, peaking in February,

following flowering in October to November. Yang et al. (2018) emphasised that climate temperature and soil moisture are key determinants of plant growth, affecting photosynthesis, flowering, and fruit ripening. Temperatures in the range observed in this study are ideal for promoting rapid growth and high yields in tropical fruit species. Moore et al. (2015) highlighted the influence of precipitation on phenology and productivity, demonstrating that seasonal variation in rainfall patterns can shape the timing of growth, overall productivity, and the composition of plant communities.

Phenology of *Artocarpus odoratissimus* based on the Extended BBCH Scale

The extended BBCH scale provides a detailed overview of the various phenological growth stages of *A. odoratissimus*. The phenological growth stages of *A. odoratissimus* outline its annual developmental process, beginning with bud development and culminating in fruit maturation (Table 1). In this study, the principal growth stages were categorised into four stages: stage 5 (reproductive development), stage 6 (flowering), stage 7 (fruit development), and stage 8 (fruit maturity). Based on the observation, *A. odoratissimus* exhibits continuous vegetative growth throughout the year as its vegetative buds develop in multiple flushes. These buds go through various developmental phases before finally transforming into leaves. As an evergreen plant, it produces vegetative shoots year-round, though vegetative growth slows during flowering and fruiting phases (Kishore, 2018). Hence, studies by Ador et al. (2024) reported that the flowering pattern and yield sometimes exhibit inconsistencies, and, according to Noor Camellia et al. (2012), this was due to weather conditions during bud differentiation affecting flower formation.

The initial reproductive phase of *A. odoratissimus* was dominated by the male phase, similar to other *Artocarpus* species, such as *A. heterophyllus* (Kishore, 2018). This male dominance ensures an abundant pollen availability when the female flowers are ready for fertilisation. The male spikes are densely covered with numerous fertile and sterile staminate flowers, with each stamen featuring an anther supported by a filament. Pollen dehiscence signifies the maturity of the male spike and the end of the male phase, which was followed by spike decay. This male phase cycle typically lasts 3 to 5 weeks.

In contrast, the female spikes emerge later on the bearing shoots. These spikes are usually smaller, rounder, and less conspicuous than the male flowers. The light green female flowers typically have thicker peduncles and bear numerous small, single flowers, each consisting of an ovary, style, and stigma. During development, the stigma emerges across the surface of the spikes and is sticky to capture pollen grains. The production of scent during spike maturation may facilitate insect attraction, thereby influencing phenological timing and maturity indices via pollination (Kishore et al., 2023). Upon successful pollination, the ovary begins to swell and develop into a syncarp.

The syncarp comprises multiple individual drupelets, each containing a seed surrounded by fleshy pulp. The female phase is completed in about 3-6 weeks.

Artocarpus odoratissimus fruits are syncarps composed of individual fertilised ovaries (achenes). Each achene develops into a bulb with a fleshy aril enclosing the ovary and a seed. The outer surface of the syncarp is covered with closely packed hairy protrusions that become more spaced out as it matures. The development of *A. odoratissimus* fruits follows a sigmoidal growth curve, characterised by an initial slow growth phase (lag phase), followed by a period of rapid exponential growth (log phase), and concluding with a phase where growth plateaus as the fruit matures (diminishing growth phase).

Fruit Development and Maturity Indices of *Artocarpus odoratissimus*

Understanding this growth pattern is essential for effectively cultivating and timing the harvest of the fruits. The lag phase spans 40 days after flower anthesis, followed by a 40-day log phase. During maturation, the fruit undergoes a period of diminishing growth lasting ~20 days (Figure 1). Fruit maturation begins after the completion of fruit development, marked by external and physical changes such as fruit and peduncle colouration, aroma emission, spacing of hairy protrusions, and reduced or absent latex production from the fruit. Fruit ripening takes 15 to 20 days. Once the fruit reaches its optimal harvesting period,

Table 1
The description of the phenological growth stages of Artocarpus odoratissimus *based on the extended BBCH scale*

Stage	BBCH Code	Description	Growth Stages	BBCH Code	Description	Growth Stages
5	**Development of male (A) and female (B) reproductive buds**					
	513A	Beginning of bud development (male)		513B	Beginning of bud development (female)	
	519A	Bract separation and male spike visible		519B	Bract separation and female spike visible	

Table 1 (*continued*)

Stage	BBCH Code	Description	Growth Stages	BBCH Code	Description	Growth Stages
6	**Flowering of male (A) and female (B) (30 days)**					
	610A	Beginning of male spike developing		611A	Beginning of male spike maturation	
	615A	Pollen dehiscence started		619A	Beginning of spike decay	
	610B	Beginning of female spike developing		611B	Beginning of female spike maturation	
	613B	Advanced maturation of female spike		615B	Spike at peak stigma receptivity stage	
	619B	Fruit set				
7	**Fruit development (~70 days)**					
	710	Syncarp begins to swell		711	Syncarp growing	
	713	30% of final fruit size		715	50% of final fruit size	
	717	70% of final fruit size				

Table 1 (*continued*)

Stage	BBCH Code	Description	Growth Stages	BBCH Code	Description	Growth Stages
8	**Fruit maturation (~15-20 days)**					
	811	Beginning of fruit maturation		815	Advanced fruit maturation	
	817	Fruit fully mature for picking		819	Fruit over mature	

it tends to become overripe if not consumed or processed immediately. The findings indicate that *A. odoratissimus* fruit reaches full developmental growth around 70 days after anthesis (AA). The fruit begins to mature at approximately 80 days AA and reaches full ripeness by around 95 days AA. However, fruits harvested after ~100 days AA are considered overripe.

Locals usually used several observational and sensory cues to determine the maturity indexes of *Artocarpus* fruits, such as colour, texture, and aroma (Elevitch & Manner, 2006). However, due to the height of the trees, the lack of distinctive colouration in the fruit, and the uncertainty about which fruits emit the characteristic aroma of ripeness, assessing factors such as texture, aroma, and external colouration is challenging, and the fruit may not exhibit clear visual cues of ripeness. To determine the appropriate maturation stage and harvesting time of *A. odoratissimus*, data on the general properties of the fruits were examined in detail in this study.

The fruit develops on day 10 AA of the flower. During the initial stage, there is rapid expansion in both fruit size and weight, primarily driven by rapid cell differentiation and enlargement. However, as the fruit progresses towards maturity, around day 80 AA, the growth rate gradually slows. At this stage, the fruit shifts its focus to maturation and ripening. The development of the pulp and seeds also occurred during this period, significantly contributing to the fruit's overall weight gain during ripening.

The findings revealed that *A. odoratissimus* fruits typically take 90 to 95 days after anthesis to reach full ripeness. This duration is similar to that of breadfruit and jackfruit, which typically require 98 and 100 days, respectively, to ripen fully (Carrington et al., 2011; Saha et al., 2016). It is noteworthy that fruit maturity is influenced by a combination of factors, including genetic makeup, the physiological condition of the specific crop, and environmental conditions (Bekele, 2018). These elements collectively play a crucial role in determining the timeline for fruit maturation. Figure 2 illustrates the developmental stages of *A. odoratissimus* fruit.

Three fruiting stages of *A. odoratissimus* have been identified in the present study: stage 1: fruit initiation, stage 2: fruit expansion and maturation, and stage 3: ripening. During stage 1, fruit initiation begins at the fruit set, marked by rapid cell division and expansion, resulting in considerable growth in length and diameter between days 10 and 20 AA. Stage 2 is known as fruit expansion and maturation. This growth continues until reaching maximum dimensions. By 60 days AA, the fruit's weight increases rapidly, reaching half its final weight by 70 days AA. During this stage, the arils expand, forming the edible flesh enveloping the seeds.

The clustered arrangement of arils within the fruit contributes significantly to its overall appearance and texture. The third stage, ripening, involves the softening and colouration of the fruit, along with the accumulation of soluble solids and acids. The flesh attains its maximum size and weight during this phase. Initially green, the fruit turns yellow or brownish-yellow as it ripens, accompanied by softening of the outer skin. Moreover, protuberances become more pronounced during ripening, while the fruit emits a sweet, fragrant aroma that intensifies with maturity. Ripening increases sugar content, resulting in a

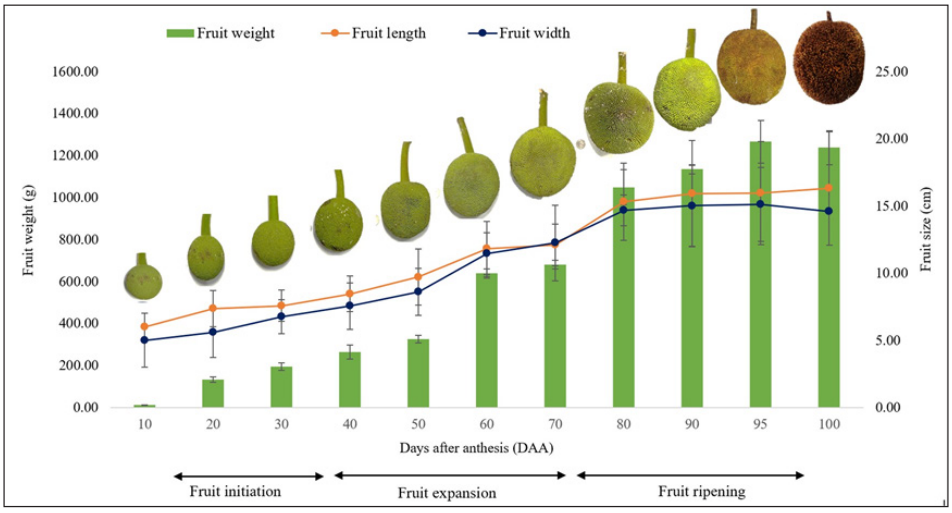

Figure 2. Fruit development of *Artocarpus odoratissimus* based on weight, length, and width

sweeter taste, and the flesh softens and becomes juicier, thereby enhancing its appeal. These collective transformations characterise ripe fruit, making it palatable and enjoyable to eat. Identifying the optimal harvesting stage is crucial for *Artocarpus* fruit. Typically harvested manually, it falls on the farmer to determine when the fruit has reached the appropriate level of maturity for harvest. The maturity of harvested produce significantly influences its shelf life and quality during storage, transportation, and marketing (Budhathoki et al., 2022). Thus, having a comprehensive understanding of maturity and employing reliable measurement techniques is imperative for *A. odoratissimus*. Ideally, the maturity index should be non-destructive and based on factual criteria rather than arbitrary standards, providing accurate insights into the commodity's quality and postharvest longevity (Reid & Jiang 2012). This holistic approach ensures that the harvested fruit meets quality standards and maintains its appeal throughout the supply chain. To determine the optimal harvest stage, maturity indices should be non-destructive and objective and should directly contribute to the quality of the commodity and its post-harvest shelf life (Reid & Jiang 2012). Data on the chemical properties of *A. odoratissimus* between 80 and 100 days after anthesis (DAA), corresponding to the maturation stage (Stage 8), are presented in Table 2. The fruit's colour, firmness, °Brix, and pH values were analysed during this period. The colour of the fruit at four different maturity stages was measured using L^* (lightness), a^* (redness), and b^* (yellowness) values. The L^* significantly differed across the stages ($p<0.05$), ranging from 42.93±2.58 at the immature stage to 24.20±2.79 at the overripe stage, decreasing as the fruit matured. The a^* value also showed a declining trend, ranging from -0.90±1.57 in immature fruits to 6.50±0.53 in fully ripened fruits. Conversely, the b^* value increased as the fruit matured, ranging from 17.80±1.47 in immature fruits to 27.77±2.03 in ripened fruits. Visual colour observations revealed that fruits harvested at 80 DAA (immature stage) were light green. At the mature stage (90 DAA), the fruit turned greenish yellow. By the ripened stage, the fruit displayed a brownish yellow to brown colour, while overripe fruits appeared dark brown.

As for the flesh, the lightness (L^*) of the flesh decreased significantly from 72.78±0.83 in immature fruits to 45.47±1.04 in overripe fruits. The a^* value, indicative of redness, exhibited a slight increase from 1.37±0.30 in immature fruits to 1.57±0.15 in fully ripe fruits. Similarly, the b^* value, representing yellowness, increased from 22.03±0.87 in immature fruits to 27.03±0.85 in ripened fruits. Visual colour observations indicated that fruits harvested at 80 days after anthesis (DAA), corresponding to the immature stage, exhibited a clear white appearance. At the mature stage (90 DAA), the fruits developed a slightly whitish yellow. During the ripened stage, the colouration transitioned to a balanced mix of white and yellow, eventually becoming fully yellowish. Overripe fruits displayed a progression from a slightly brownish tint to a fully brown colouration.

Table 2
Physicochemical properties of Artocarpus odoratissimus *fruit based on different stages of maturity*

Variables	Day 80 AA (Immature)	Day 90 AA (Mature)	Day 95 AA (Fully ripen)	Day 100 AA (Over-ripen)
Peel				
L*	42.93±2.58^{a} (40.60-45.70)	32.43±3.57^{b} (29.80-36.50)	27.75±0.93^{bc} (26.70-28.40)	24.20±2.79^{c} (21.00-26.10)
a*	-0.90±1.57^{c} (0.80-(-2.30))	1.60±0.20^{bc} (1.40-1.80)	3.50±0.56^{b} (3.00-4.20)	6.50±0.53^{a} (5.90-6.80)
b*	17.80±1.47^{c} (16.50-19.40)	21.40±0.95^{bc} (20.30-22.00)	23.90±1.47^{ab} (22.30-25.20)	27.77±2.03^{a} (25.50-29.40)
Visual	Score 1 Light green	Score 2 Greenish yellow	Score 3-4 Brownish yellow to brown	Score 5 Dark brown
Flesh				
*L**	72.78±0.83^{a} (71.70-74.40)	63.30±1.04^{b} (61.40-65.00)	56.63±0.84^{c} (55.10-58.00)	45.47±1.04^{d} (43.70-47.30)
a*	1.37±0.30^{ab} (0.80-1.80)	1.03±0.23^{b} (0.60-1.40)	1.93±0.09^{a} (2.10-1.80)	1.57±0.15^{ab} (1.80-1.30)
b*	22.03±0.87^{b} (20.80-23.70)	26.47±0.59^{a} (25.30-27.20)	26.30±0.60^{a} (25.10-27.00)	27.03±0.85^{a} (25.90-28.70)
Visual	Score 0 White	Score 1 Slightly whitish yellow	Score 2-3 Equal white yellow to Fully yellowish	Score 4-5 Slightly brownish to fully brown
Firmness (N)	12.10±0.10^{a} (11.20-11.60)	6.90±0.10^{b} (6.80-7.00)	4.00±0.10^{c} (3.90-4.10)	0.90±0.10^{d} (0.80-1.00)
Total soluble solid (°Brix)	13.20±0.26^{c} (13.00-13.80)	17.60±0.30^{b} (17.30-17.90)	24.03±0.15^{a} (23.90-24.20)	24.50±0.10^{a} (24.20-24.60)
pH	4.33±0.06^{c} (4.60-4.70)	4.97±0.06^{b} (4.90-5.00)	5.33±0.12^{a} (5.20-5.40)	4.40±0.10^{c} (4.40-4.60)

Note. Different superscript alphabet in the same row indicates differences at $p<0.05$ (ANOVA, Tukey's test). Values are given as means±standard deviation, and values in parentheses are the range

For the fruit firmness, on day 80, *A. odoratissimus* was recorded at 12.10±0.10 N. By day 90, as the fruit neared ripeness, firmness decreased to 5.90±0.10 N and further declined to 4.00±0.10 N by day 95, indicating full ripeness. By day 100, firmness dropped sharply to 0.90±0.10 N, signalling over-ripeness. This pattern aligns with findings from a study on *A. heterophyllus* by Ranasinghe and Marapana (2019). According to a study by Ranasinghe and Marapana (2019), the firmness of *A. heterophyllus* gradually decreases as it approaches ripeness, with a more rapid decline during ripening and over-ripening stages.

Table 3 presents the proposed maturity-based phenological framework that supports diversified utilisation pathways for *A. odoratissimus* fruit. Harvesting before 80 days after anthesis (AA) was not recommended due to the presence of densely packed hairy protuberances, incomplete flesh and seed development, and excessive latex production. *Artocarpus odoratissimus* fruits harvested at day 80 were considered suitable for cooking. Additionally, fruits at day 90 AA can be harvested and stored at room temperature to ripen, as this fruit is known to be climacteric (Ismail et al., 2023). Meanwhile, fruits harvested on day 95 AA are preferred for fresh consumption, as they are naturally ripened and sweeter compared to those harvested on day 90. However, by day 100, the fruits are considered overripe, with a darkened peel and an excessively soft texture. Although the flesh remains edible, it often develops a slightly tangy or unpleasant aroma, signalling the onset of over-ripeness.

Table 3
Proposed landmark phenological stages with implications for the cultural and culinary practices of Artocarpus odoratissimus *fruit based on different stages of maturity*

Landmark Phenological Stages	Description	Implication for Cultural and Culinary
Beginning of fruit maturation	Fruits harvested at day 80 after anthesis	*Artocarpus odoratissimus* fruits harvested at day 80 were considered suitable for cooking, which is a common trait observed in other Artocarpus species, such as breadnuts and jackfruit. Immature fruits from these species are often sliced and cooked as vegetables, providing a versatile and nutritious food source. Young jackfruit is popular in many cuisines, where it is cooked as a meat substitute because of its texture and nutrient density.
Advanced fruit maturation	Fruits harvested at day 90 after anthesis	Fruit at this stage is suitable to be harvested and stored at room temperature for ripening, as this fruit is known to be climacteric in nature or suitable for transport.
Fruit fully matures for picking	Fruits harvested on day 95 after anthesis	Fruits are naturally ripened and sweeter. The flesh can be eaten raw, cooked into fritters, or fermented to make organic vinegar and ice cream flavour. As for the seeds, they can be boiled or steamed and eaten as a snack. It can also be processed to make flour.
Fruit fully matures for picking	Fruits harvested on day 100 after anthesis	Overripe-darkened peel and excessively soft texture. The flesh remains edible, often develops a slightly tangy aroma, signalling the onset of over-ripeness.

Regarding TSS, reflecting mineral and sugar content in fruits and expressed in °Brix, pulp TSS increased gradually over time from 13.20±0.26 °Brix on day 80 to 24.50±0.10 °Brix on day 100, suggesting starch breakdown into sugars during later harvesting stages and rapid metabolic transformations. Similar increasing TSS trends toward maturity were noted in studies by Rana et al. (2018) and Ranasinghe and Marapana (2019) in *Artocarpus*. The pH slightly rose from 4.33±0.06 on day 80 to 5.33±0.12 on day 95, then decreased to 4.40±0.10 on day 100, indicating slight acidity in over-ripened fruits and suggesting chemical deterioration. During ripening, the slight decline in pH is attributed to the degradation of organic acids, which are either converted into sugars or utilised in respiration (Richard et al., 2025). At the overripe stage, senescence and cell wall degradation compromise membrane integrity, resulting in the release of organic acids and the formation of acidic metabolites (Kong & Singh, 2016).

Additionally, there is a slight increase in pH during the ripening process, followed by a decrease in acidity in over-ripened fruits. These findings suggest that shared physiological and biochemical processes underlie the ripening and maturation of Artocarpus species, despite differences in specific characteristics and flavours. This indicates a common ripening pattern across *Artocarpus* species, highlighting the importance of understanding these shared processes in fruit development and quality.

Limitations

Several limitations should be considered when interpreting the findings of this study. The proposed harvest windows should be applied under typical rainfall and temperature conditions, prioritising phenological staging over days after transplanting alone. The BBCH framework, when combined with key maturity indices, provides an adaptive approach to harvesting decision-making. Under unusual rainfall, the fruit development rates may alter. Therefore, confirmation of harvest readiness at BBCH stages 815-817 using instrumental measurements is recommended rather than relying solely on days after anthesis. Additionally, although the BBCH framework provides a transferable and standardised description of fruit development stages, calendar-based timing and threshold values may vary across locations; therefore, local calibration under different microclimatic conditions and management practices is recommended. Furthermore, variations in soil fertility and nutrient management may influence flowering time and fruit development rates; consequently, the phenological events reported in this study may differ across different soil and management regimes.

CONCLUSION

This study highlights the significance of understanding the phenological growth stages of *A. odoratissimus* using the extended BBCH scale, which provides a structured framework

for identifying key developmental phases. Fruit maturation occurs in three distinct stages, with the optimal harvesting periods determined as day 90 after anthesis (AA) for storage and day 95 AA for immediate consumption. These findings fill a critical knowledge gap in *the phenology of A. odoratissimus*, offering practical insights to refine harvesting practices. By equipping local farmers with a valuable tool to optimise yields and sustainably manage production, this study contributes to the broader commercialisation of the fruit. Future research should explore the environmental and agronomic factors influencing its phenology and quality to further support sustainable production and market expansion.

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CONFLICTS OF INTEREST

There is no conflict of interest.

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Enhancing the Physical Properties of Egg Custard-based Diets through Feed Supplements to Improve the Growth Performance of *Macrobrachium rosenbergii* Larvae

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ABSTRACT

The giant freshwater prawn, *Macrobrachium rosenbergii*, is an important species in freshwater crustacean aquaculture. Traditional larval feeding methods relying on egg custard are challenged by poor buoyancy and significant nutrient leaching. This study aims to enhance the physical properties of egg custard-based diets by incorporating carboxymethyl cellulose (CMC) and buoyancy modifiers (BM, yeast, and baking powder). Three experimental diets were formulated with varying CMC and BM levels (D1: 5% CMC; D2: 2% CMC & 3% BM; D3: 0% CMC & 5% BM) and evaluated on floatability, water stability, and nutrient retention. The study also involved feeding larvae with these diets and assessing growth performance over several weeks. Assessing the physical properties of each diet over 60 min found that D3 was the most buoyant, achieving a flotation rate of 49.24%, while D2 had a rate of only 32.98%, and D1 20.90%. Water stability assessments showed that D1 retained 70.80% stability after 40 min, compared to 69.55% for D2 and 86.61% for D3. The nutrients of nutrient leaching were not too dissimilar: D1 had a protein retention of 89.00%, D2 a retention at 87.60%, and D3 86.61%. The lipid retention of each diet followed a similar trend, with D1 retaining 90.21% of its lipid content, D2 88.49%, and D3 at 84.47%. This study highlights the importance of optimising dietary formulations for *M. rosenbergii* larvae, demonstrating that balancing buoyancy and nutrient retention can lead to improvements in the growth and survival of larval cultures. The study concludes that incorporating CMC and BM can enhance diet quality and larval performance.

Keywords: Aquaculture, feeding strategy, growth performance, *Macrobrachium*, nutrient leaching, water stability

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INTRODUCTION

The aquaculture of the giant freshwater prawn, *Macrobrachium rosenbergii,* is economically significant, ranked as the fourth most important crustacean species, in terms of tonnage, globally (Wahl et al., 2023). In the hatchery phase of production, the larvae are traditionally fed live *Artemia* nauplii and egg custard (Taguemount et al., 2024). While *Artemia* are highly mobile, the egg custard particles do not move, and these must be caught by chance as they pass the prawn larvae; this property of egg custard may potentially hinder the successful growth and survival of larvae (Taguemount, 2025). Although the egg custard, made from chicken eggs and skimmed milk, is easily digestible, its tendency to sink makes it unsuitable for *M. rosenbergii* larvae, which feed in the water column. Additionally, the high moisture content of the egg custard results in low water stability and significant nutrient leaching (Dhont et al., 2010). Typically, shrimp feed contains 10-40% fishmeal and 9-20% soybean meal, though the reliance on fishmeal is declining due to the cost of these ingredients and their sustainability (Kasamechotchung et al., 2025; Nunes et al., 2022). The inclusion of fish and squid oils (3%) enhances feed attraction (Yusoff et al., 2021), while starches (10-20%) serve as energy sources and binders (Munkongwongsiri & Poolsawat, 2024; Tinh et al., 2021). Other dietary ingredients include fibre, vitamins, and minerals (Ayisi et al., 2017; Nesara & Paturi, 2018); effective formulations must consider both the nutritional composition and the physical properties of the diet. Additives such as cellulose (1-3%) help improve buoyancy and stability, while binders like carboxymethylcellulose (CMC) and agar help enhance feed integrity (Ayuhafizah et al., 2024). The incorporation of *Rhizopus oligosporus* mycelium can also enhance structural stability and buoyancy (Nurohmah et al., 2023).

The stability and floatability of the aquatic diet, therefore, are crucial for prawn feed. Ideal feed should maintain buoyancy, settle slowly, and minimise nutrient loss, thereby accommodating the continuous feeding habits of the larvae (Jannathulla et al., 2019). Water stability refers to pellet integrity and minimal disintegration (Karim et al., 2022), while the floatability can help determine the suitability of a diet for a particular feeding strategy (Irungu et al., 2018). The bulk density is closely related to the sinking velocity of feed, with high-density particles sinking faster (Jannathulla et al., 2019). Understanding the sinking and leaching rates is important as this knowledge can lead to optimised feeding regimes (Zaabwe et al., 2020). For the larviculture of *M. rosenbergii*, encapsulation is not a viable strategy for high-moisture feeds (>50%), so a practical approach to this is to include specific ingredients to enhance buoyancy and to reduce nutrient leaching (Aaqillah-Amr et al., 2021).

A wide range of different ingredients and techniques have been explored to improve the physical properties of aquafeeds. Binders, for example, help maintain diet integrity and cohesion when in water (Miladinovic, 2020), with carboxymethyl cellulose (CMC) being

widely recognised as a cost-effective binder that improves pellet stability (Rahman et al., 2021). For *M. rosenbergii*, optimal feed stability has been reported with inclusion rates of 2% to 4% CMC (Aaqillah-Amr et al., 2021; Yusoff et al., 2021). Other method to improve feed buoyancy include incorporating air into the feed matrix, adjusting oil levels, and using upwelling currents during feeding (Kolkovski, 2004). The use of density control agents can reduce feed density, aiding flotation (Kari et al., 2023), while fungal fermentation with oven drying has also been shown to improve water stability and floatability (Sriherwanto et al., 2021). The carbohydrate content of diets can also influence these properties; for example, guinea corn has been reported to outperform maize and wheat in producing floating pellets (Fashina et al., 2019). The combination of wheat offal with yeast has also been shown to provide superior expansion and floatation characteristics (Momoh et al., 2016). Gelatin, CMC, and guar gum have been demonstrated to enhance water stability and growth performance across various species (Ayoola, 2020; Gao et al., 2020).

The use of yeasts and baking powder represents another approach to enhancing pellet buoyancy (Suleiman et al., 2008). Yeast contributes to buoyancy through the production of carbon dioxide (CO_2) during fermentation (Tofalo et al., 2020), while baking powder, a leavening agent, comprised of sodium bicarbonate (baking soda) and an acid component, typically phosphate salts, releases CO_2 on contact with moisture. This release of CO_2 aerates the feed matrix and contributes to its buoyancy (Kollemparembil et al., 2023). Orire and Sadiku (2014) found that diets incorporating 10% of these ingredients, i.e., yeast and baking powder, had a flotation of approximately 70% after 60 minutes. When the inclusion of these was reduced to 5%, 60% of both pellets and flakes remained afloat after 60 minutes, suggesting adequate buoyancy. Standard egg custard particles sink and leach nutrients; therefore, the buoyancy and nutrient retention of the diet needs to be improved if it is to meet the nutritional needs of the *M. rosenbergii* larvae. This study, therefore, investigates the inclusion of CMC and buoyancy modifiers (yeast and baking powder) on the integrity and buoyancy of egg custard-based diets, and on the larviculture performance of larvae.

MATERIALS AND METHODS

Animal Husbandry

Gravid *M. rosenbergii* females (average weight of 38.05 ± 2.87 g) were collected from the Manir River, Terengganu, Malaysia (05°17′39.2″N, 103°05′23.1″E) and transported to the marine hatchery at Universiti Malaysia Terengganu, Malaysia. The berried females were housed individually in 80 L tanks, where the salinity was gradually increased to 12 ppt. The female prawns were fed twice daily using a commercial pelleted feed (40% protein, Gold Coin Pro Series, Indonesia) supplemented with fresh mussels at a rate of 5% body weight per day (i.e., an average daily feed ration of 1.90 g).

After 15 to 20 days of maintenance, the larvae hatched (primarily overnight); these were then counted and collected using a five-point method under low lighting conditions and then transferred to the larval-rearing tanks.

Experimental System and Water Management

The larviculture setup included 18 × 150 L rectangular, dark blue fibreglass tanks connected to a submerged biofilter divided into multiple chambers. Each tank was filled and maintained with 130 L of 12 ± 1 ppt seawater and a porous air stone for aeration. A gravity-fed water system circulated water from the larviculture tanks to the biological filter. An outlet screen with a mesh size of 250 µm was placed at the top of the drainpipe (2.5 mm) to allow water to flow back into the biological filter while retaining the larvae and *Artemia* within the rearing tank. An air-lift pump was used to return the filtered water to the rearing tank. To prevent water overflow, the filter screen was cleaned daily. Any water loss due to evaporation was compensated for by adding more brackish water. Each of the six experimental diets was randomly assigned to triplicate groups of larvae, starting with 7,800 larvae per tank at a stocking density of 60 larvae/L. Daily maintenance involved removing residual feed, dead larvae, and waste before the morning feeding, along with ~10 % daily water changes to maintain optimal water quality. Water quality parameters, temperature, pH, salinity, and dissolved oxygen were monitored using a YSI multiparameter meter (5908 Cap Membrane Kit, USA), with ammonia and nitrite levels recorded using a test kit (Model NI-SA, Loveland, CO, USA). The results showed an average water temperature of 28.94 ± 3.96 °C, dissolved oxygen at 6.23 ± 1.06 mg/L, pH at 8.63 ± 1.7, and salinity at 12.63 ± 1.85 ppt. Total ammonia and nitrite levels were consistently below 0.1 mg/L. A natural photoperiod of at least 12 hours daily was maintained to support larval growth.

Experimental Diets

Two experimental diets (D2 and D3) and a control (D1) were formulated with the following percentages of CMC and BM: D1 (5% CMC & 0% BM), D2 (2% CMC & 3% BM), and D3 (0% CMC & 5% BM). The BM consisted of a 50:50 mixture of yeast and baking powder. To achieve the 3% and 5% BM levels in the diets, yeast and baking powder were added in equal ratios. Therefore, the (CMC: BM) ratios were 5/0 (all CMC), 2/3 (mixed), and 0/5 (all BM) (Table 1). Each dry ingredient was individually weighed and thoroughly mixed. Chicken eggs and fish oil were then added to the mixture and blended using a Pensonic MX-GM 1011 blender for 5 minutes, ensuring complete integration of all the ingredients. The homogenate was then supplemented with activated yeast and baking powder as the buoyancy modifier. This mixture was allowed to rest at room temperature for 30 minutes to allow the yeast to activate. Then, the mixture was placed in a two-tiered stainless-steel steamer and steamed using a portable gas stove (Pensonic PPG-2002N), with the lower containing boiling water, while the upper perforated tier allowed steam to pass through and

cook the egg custard diet. This setup is commonly used for steaming food and represents a healthy cooking method that preserves nutrients (Martinez, 2024). The steaming was done at a temperature range of 75 to 100 °C for precisely 15 minutes. After steaming, the diet was allowed to cool to room temperature before further handling. The prepared diet was refrigerated at 5 °C and used within a few days of preparation to ensure freshness and nutritional integrity. The nutrient content of the experimental diets was analysed according to the methods outlined by the Association of Official Analytical Chemists (AOAC) (1995). Parameters assessed included moisture, ash, protein, lipids, and fibre (Table 1).

Table 1
Composition and nutrient analysis of the formulated experimental diets

Ingredients (g/100g dry weight)	**Control (0% BM)**	**3% BM**	**5% BM**
	D1	**D2**	**D3**
Chicken egg	47	47	47
Milk powder[1]	20	20	20
Rolled oats[2]	10	10	10
Fishmeal[3]	8	8	8
Fish oil[4]	5	5	5
Diatomaceous earth[5]	2	2	2
Carboxymethylcellulose[6]	5	2	0
Mixed vitamins[7]	1.5	1.5	1.5
Mixed minerals[8]	1.5	1.5	1.5
Buoyancy modifier[9]	-	3	5
Proximate composition[10] % dry matter			
Moisture (% of wet weight)	53.05 ± 0.51	52.37 ± 0.16	46.99 ± 2.54
Other Components (% of dry matter)			
Ash	9.2 8 ± 0.08	8.99 ± 0.10	09.13 ± 0.61
Protein	50.14 ± 0.64	50.68 ± 2.45	50.51 ± 1.65
Lipid	37.61 ± 0.33	37.79 ± 2.15	37.86 ± 2.19
Fibre	01.40 ± 0.00	01.40 ± 0.00	01.40 ± 0.00
NFE	01.65 ± 0.22	01.14 ± 0.32	01.10 ±0.18

Note. BM = buoyancy modifier. D1 (control) = 5% CMC & 0% BM; D2 (3%BM) = 2% CMC & 3% BM; D3 (5% BM) = 0% CMC & 5% BM. NFE = Nitrogen-Free Extract

[1]Nestle Products Sdn. Bhd., Petaling Jaya, Kuala Lumpur, Malaysia; [2]Scc Marketing (M) Sdn Bhd., Puchong, Selangor, Malaysia; [3]TripleNine, 700 g kg^{-1} crude protein; [4]TripleNine, 100% marine oil; [5]Global Agora Resources., Bandar Pinggiran Suban 40150 Shah Alam, Malaysia; [6]D Chemie Chemical Supplie, Skudai, Johor, Malaysia; [7]Vitamin premixes contained (as g/kg) vitamin A, 50; vitamin D3, 10; vitamin E, 130; vitamin K3, 10; vitamin B1, 10; vitamin B2, 25; vitamin B6, 16; vitamin B12, 0.1; niacin, 20; pantothenic acid, 50; folic acid, 8; biotin, 0.5; anti-caking agent, 20. DSM Nutritional Products (Thailand) Ltd 700/437 Chonburi, Thailand; [8]Mineral premixes contained (as g/kg) copper, 7.5; iron, 125; manganese, 25; zinc, 125; cobalt, 0.5; iodine, 0.175; selenium, 0.3; anti-caking agent, 10; [9] Taiwan; [10]Seafield Food Ind. Sdn. Bhd, Malaysia, and Barkath Foods Sdn. Bhd, Malaysia

Feeding Trial

From day 2 post-hatch, larvae were initially fed newly hatched *Artemia* nauplii at 1-5 nauplii/mL four times daily (10:00, 14:00, 18:00, and 23:00 h). Starting on day 7 (stage 6 to PL), the diet included *Artemia* nauplii and various formulated egg custard diets: control (5% CMC & 0% BM), D2 (2% CMC & 3% BM), and D3 (0% CMC & 5% BM). Larvae were fed 0.2-0.3 g of the relevant egg custard diet for days 8-15 and 0.3-0.5 g for days 16-28 per 1000 larvae (Murthy et al., 2008). Feed particles were manually sieved (pore size ~ 400 -1000 µm), then mixed with culture water (20 mL), and fed using a Pasteur pipette to ensure larvae could catch feed particles. Feeding of prepared feed occurred three times a day during daylight hours (10:00, 14:00, and 18:00 h), and once overnight at 23:00 hour with *Artemia* nauplii.

Evaluation of the Physical Properties of the Experimental Diets

Morphology of Feed Particles

The surface morphology of the egg custard feed particles was examined using a scanning electron microscope (3rd Generation VEGA SEMs, Tescan, Czechia). For sample preparation, the diets were thoroughly dried at 40°C to remove moisture. Particles of each diet were then mounted on the SEM sample holder using double-sided carbon tape. Subsequently, the samples were sputter-coated with a thin layer of gold (approximately 10-20 nm thick) in an argon atmosphere to enhance electrical conductivity and prevent charging during electron beam exposure (Anas et al., 2008). The surface morphology of each diet was then examined to assess the influence of each inclusion of CMC and BM.

Floatation Test

The floatability of the egg custard feed particles incorporating a buoyancy modifier (BM) was assessed (Equation 1) under the standard rearing conditions for prawn larvae (12 ± 1 ppt salinity; 27 ± 1°C). Formulations tested included D1 (5% CMC & 0% BM), D2 (2% CMC & 3% BM), and D3 (0% CMC & 5% BM). Before testing the properties of each diet, the aeration and water circulation were stopped to accurately measure floatability. Based on a stocking density of 7,800 larvae per tank and a feeding rate of 0.325 g of feed per 1,000 larvae per day, the total quantity of daily feed was calculated to be 2.535 g. Three feeds were given each day (0.845 g per feeding). During each flotation test, 0.0845 g (10% of 0.845 g) of each diet was added to a floating ring representing 10% of the water surface area (300 cm^2 of 3,003 cm^2). The average number of particles in 0.0845 g was 22.33 ± 0.58 for D1, 31.33 ± 1.53 for D2, and 44 ± 1.73 for D3. Each assessment lasted 60 min, with the number of pellets recorded at 10-min intervals across triplicate tanks (18 replicates per feed type).

$$\text{Floatation rate (\%)} = (\text{Final no. of particles afloat}) / (\text{Initial no. of particles afloat}) \times 100 \quad [1]$$

Water Stability and Dietary Nutrient Leaching

To determine the water stability (Equation 2), a 5 g sample of each experimental diet (D1 contained 5% CMC & 0% BM, D2: 2% CMC & 3% BM, D3: 0% CMC & 5% BM) were placed on 0.1 mm mesh nylon filter cloths and immersed in the larviculture tanks (130 L, 12 ± 1 ppt salinity, 27 ± 1°C) containing no prawn larvae. Each mesh was secured to prevent physical damage during immersion. Representative samples of each diet were removed after 10, 20, 30, and 40 min, then air-dried, and weight loss was determined by comparing initial and post-immersion weights (Kovalenko et al., 2002). All measurements were conducted in triplicate. Following immersion, the protein (Equation 3) and lipid content (Equation 4) of each sample of diet was analysed using the Kjeldahl and Soxhlet extraction methods, following AOAC (1995) protocols.

$$\text{Water stability (\%)} = (\text{initial feed weight-dried feed weight (g)}) / (\text{initial feed weight (g)}) \times 100 \quad [2]$$

$$\text{Protein retention (\%)} = (\text{Post-immersion protein content}) / (\text{Initial protein content}) \times 100 \quad [3]$$

$$\text{Lipid retention (\%)} = (\text{Post-immersion lipid content}) / (\text{Initial lipid content}) \times 100 \quad [4]$$

Evaluation of Larval Development and Growth Parameters

The larval development of the *M. rosenbergii* larvae was determined using the descriptions provided by Uno & Kwon (1969), following their development every alternate day assessed by using an Olympus STM7 dissecting stereo microscope. The larval stage index (LSI) was calculated using the method by Maddox and Manzi (1976): LSI = (∑ Sixni)/N, where *S* is the larval stage, *ni* is the number of larvae in each stage, and N is the total number of larvae examined. Upon conclusion of the experiment, the survival rate, metamorphosis rate, rearing period, and average dry weight of the larvae were determined. Metamorphosis

(recorded by days) was recorded when 95% of the larvae had become post-larvae (PL). Survival and metamorphosis rates were calculated using Equation 5 and Equation 6

$$\text{Survival rate (\%)} = (\text{number of post-larvae produced} / \text{number of stocked larvae}) \times 100 \quad [5]$$

$$\text{Metamorphosis rate (\%)} = (\text{final number of post-larvae} / \text{numbers of stocked larvae}) \times 100 \quad [6]$$

For post-larval growth, 50 individuals from each treatment group were sampled and euthanised in iced water. Total length (TL, from rostrum to telson) was measured using digital callipers (± 0.1 cm), and dry weight (DW, mg) was determined by oven-drying at 60°C for 24 hours, then weighing with AT21 Mettler Toledo, Inc. (Shanghai, China; 1 μg).

Statistical Analysis

The obtained physical properties of the feed, namely flotation rate, water stability, protein, and lipid retention rates, were subjected to a two-way ANOVA. All proportion data were arcsine transformed before analysis. The variation of these features for each feed type over time was analysed using one-way ANOVA. The growth performance data (i.e., survival rate, body length, dry weight and metamorphosis rate, etc.) were evaluated using one-way ANOVA. Where significant differences were found, Duncan's test was used to identify significant differences between the three experimental diets. Statistical analyses were performed using the SPSS version 26.0.

RESULTS AND DISCUSSION

Morphology of Feed Particles

From Figure 1, diet D1 (0% BM & 5% CMC) (Figure 1A) appeared to be more compact. The high content of the binder likely results in a more uniform and cohesive structure with fewer and smaller pores. In contrast to this, diet D2 (3% BM & 2% CMC) (Figure 1B) possessed a rough texture with numerous holes of varying sizes; the binder provided cohesion, the buoyancy modifier helping to create more pores. Lastly, feed D3 (5% BM & 0% CMC) (Figure 1C) displayed a complex porous structure resembling a sponge or coral. The high buoyancy modifier content created more open and interconnected pores, while the absence of the binder resulted in less cohesion, leading to a more open and interconnected structure.

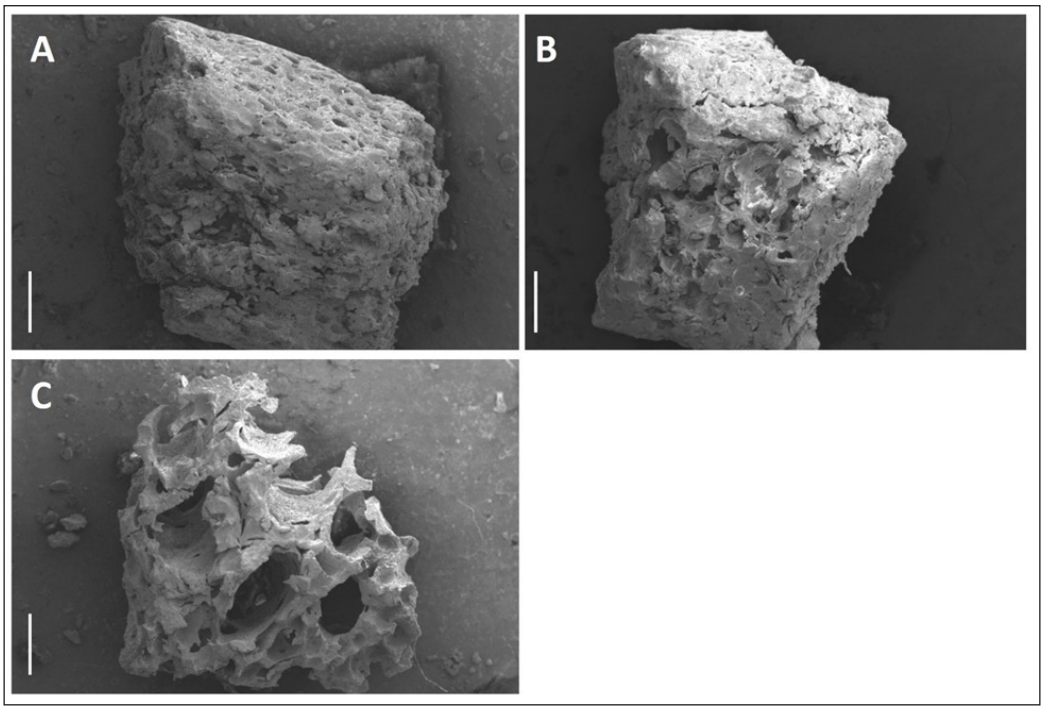

Figure 1. Scanning electron micrographs of representative egg custard particles from each of the three experimental diets. A. Diet D1 with 5% CMC and 0% BM; B. Diet D2 with 2% CMC and 3% BM; C. Diet D3 with 0% CMC and 5% BM. Scale bar = 1 mm

Floatability

The flotation analysis (Table 2) indicated significant effects of both groups ($F(2, 36) = 28.922$, $p < 0.001$) and time ($F(5, 36) = 7.703$, $p < 0.001$) on flotation, with no significant interaction between groups and time ($F(10, 36) = 1.657$, $p = 0.130$). This suggested a consistent effect of time on flotation rates across all diets. D1, D2, and D3 exhibited statistically significant differences in flotation rates ($p<0.05$), following the trend D1 < D2 < D3 (Fig. 2A). The flotation rate gradually declined over time with no significant differences between 10-40 minutes ($p>0.05$), there was, however, a significant decrease from 50 to 60 minutes ($p<0.05$) (Fig. 2B). Proportional flotation showed no significant variations ($p>0.05$) across timepoints, peaking at 20 minutes and declining at 60 minutes for D1. D2 showed a significant decrease by 30 minutes ($p<0.05$) and continued to decline until 60 minutes. D3 remained stable from 10 to 50 minutes, with a significant drop at 60 minutes ($p<0.05$), as shown in Fig. 2C. These results showed that while each experimental diet had different rates of flotation, the general trend over time was similar.

Buoyancy in aquaculture feeds is an important feature that optimises feed accessibility and directly influences the growth and survival of each species (Onomu & Okuthe, 2024).

of the three experimental diets, the control diet (D1, 5% CMC & 0% BM) had the lowest flotation rate after 60 minutes (20.90%, $p<0.05$) (Figure 2A). CMC, a binder, improves pellet integrity and stability, resulting in higher-density particles with a faster sinking rate when used alone at 5% (Attar et al., 2018). The inclusion of 3% BM in D2 and 5% BM in D3 significantly improved the buoyancy of the diets compared to D1, with D3 showing the highest overall flotation rate (Figure 2A, $p<0.05$).

The improved buoyancy of D2 and D3 is linked to the use of yeast (*S. cerevisiae*) as a buoyancy modifier. During fermentation, the yeast produces CO_2, which gets trapped in the feed matrix, creating air-filled spaces that improve buoyancy (Struyf et al., 2017). Similarly, baking powder acts as a chemical leavening agent, releasing CO_2 gas when mixed with moisture and heat, aerating the feed matrix and creating a porous, low-density diet (Gélinas, 2024; Luck, 2021). Together, these ingredients and mechanisms significantly enhance the buoyancy of the diet, allowing it to float effectively in the water column (Orire & Sadiku, 2014). These agents create larger voids within the pellet structure, leading to a porous structure that traps air and results in lighter feed particles (Figure 1B, C).

Flotation rates decreased over time, showing no significant differences between 10 to 50 minutes but a notable decline at 60 minutes (Figure 2B, C). The interaction between dietary groups and time was not significant. The decrease in flotation over time is most likely due to water absorption, and/or feed disintegration

Table 2
The results of a two-way ANOVA assessing three experimental freshwater prawn diets and their interaction on their floatability, water stability, immersion time (measured at 10 min intervals from 10 to 60 min) and capacity for retaining their protein and lipid content. The three diets differed in their inclusion rate of buoyancy modifiers (BM) and carboxymethyl cellulose (CMC, where: D1 (control; 5% CMC; 0% BM); D2 (2% CMC; 3% BM); and D3 (0% CMC; 5% BM). F = F-ratio, and Sig = significance at p≤0.05

Source	Floatability		Stability		Protein Retention		Lipid Retention	
	F	Sig.	F	Sig.	F	Sig.	F	Sig.
Corrected Model	6.643	<0.001	24.818	<0.001	35.275	<0.001	49.138	<0.001
Groups	28.922	<0.001	40.895	<0.001	1.342	0.28	29.459	<0.001
Time	7.703	<0.001	58.719	<0.001	123.572	<0.001	156.636	<0.001
Groups × Time	1.657	0.13	2.509	0.05	2.437	0.06	1.948	0.11

Note. R Squared and Adjusted R Squared:
Floatability: 0.758 (Adjusted R Squared = 0.644)
Stability: 0.919 (Adjusted R Squared = 0.882)
Protein Retention: 0.942 (Adjusted R Squared = 0.915)
Lipid Retention: 0.957 (Adjusted R Squared = 0.938)
Groups and Time Points:
Groups: D1 (5% CMC; 0% BM), D2 (2% CMC; 3% BM), D3 (0% CMC; 5% BM)
Time Points: 10, 20, 30, 40, 50, and 60 minutes

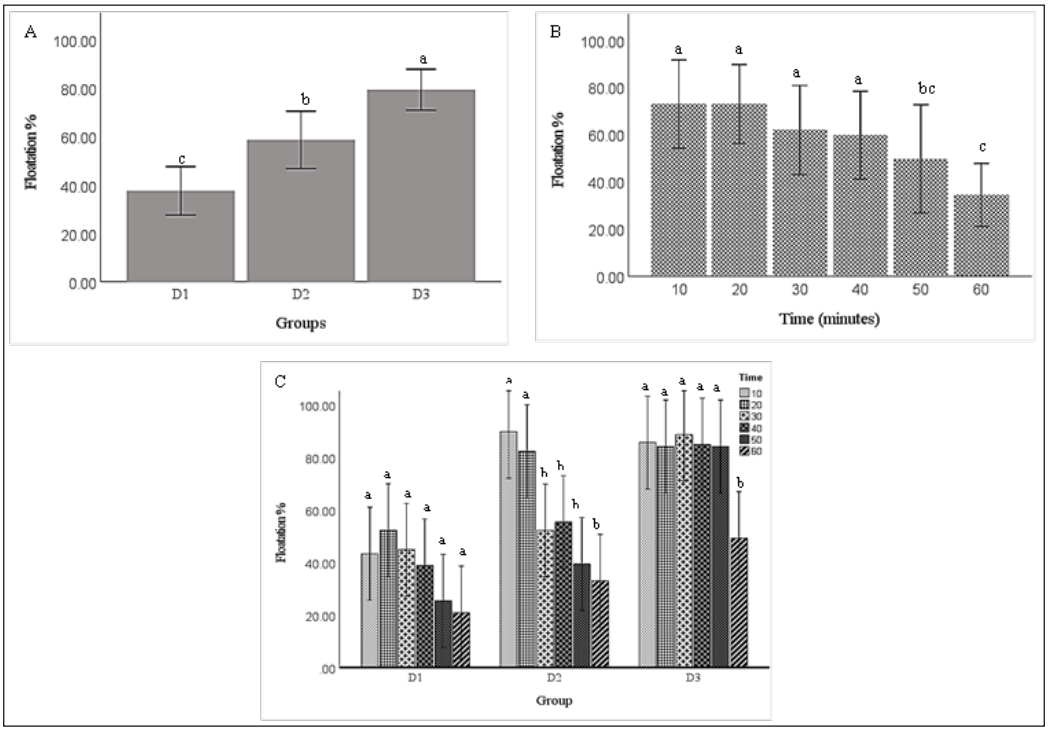

Figure 2. Flotation performance of the three experimental diets (D1-D3); A. Flotation ratios for each diet over a 60-minute period, where D1 = 5% CMC & 0% BM; D2 = 2% CMC & 3% BM, and D3 = 0% CMC & 5% BM); B. The mean flotation ratios averaged across the three experimental diets, without differentiating between diet group or composition, across the 60-minute assessment period; C. Flotation percentages for each diet were determined at 10-minute intervals over the 60-minute assessment. Error bars represent standard deviations of three replicates; different letters indicate statistically significant differences in flotation performance at $p \leq 0.05$

(Chen et al., 2010; October et al., 2019). Lower bulk density, achieved by using lighter ingredients and buoyancy-enhancing formulations, contributes to better floatability in aquafeeds (Ma et al., 2020; Patel et al., 2024; Soares et al., 2021). These studies are consistent with previous studies confirming the use of yeasts and baking powder as buoyancy modifiers, which create larger voids and trap air within feed particles (Adekunle et al., 2014; Falayi & Sadiku, 2013). For example, Adekunle et al. (2014) reported floatation rates of up to 70% using these catalysts, while other binders like cassava starch and Aquatec-11™ achieved similar results (Ma et al., 2021; Orire & Emine, 2019). These results align with the current study, where BM agents are crucial for achieving buoyancy in diets formulated for *M. rosenbergii* larvae. Ensuring 80-100% buoyancy is generally accepted to facilitate efficient feeding behaviour, which is critical for species like *M. rosenbergii*, which are slow feeders (Assan et al., 2021).

Water Stability and Dietary Nutrient Leaching

Table 2 shows that both diet ($F(2, 24) = 40.895$, $p<0.001$) and time ($F(3, 24) = 58.719$, $p<0.001$) significantly affected water stability, with no significant interaction between groups and time ($F(6, 24) = 2.509$, $p = 0.050$). This indicates consistent effects of diet on stability across time intervals. Significant differences were observed between diets over time ($p<0.05$), following the trend D1 > D2 > D3 (Fig. 3A). Water stability progressively decreased over time at each timepoint ($p<0.05$) (Fig. 3B). Within each group, no significant differences were observed from 10 to 40 minutes ($p>0.05$) despite a steady decline. Group D2 showed a similar trend, decreasing to 73.60 ± 0.46 at 10 min and to 69.55 ± 0.35 at 40 minutes. Group D3's stability declined to 72.74 ± 1.27 at 10 min and further to 68.61 ± 0.33 at 40 minutes (Figure 3C).

The findings from Table 2 indicate that time significantly affected protein retention ($F(3, 24) = 123.572$, $p<0.001$), while the type of diet did not ($F(2, 24) = 1.342$, $p = 0.281$). The interaction between group and time was also not significant ($F(6, 24) = 2.437$, $p = 0.055$), implying that the impact of diet on protein retention remained consistent across different time intervals. No significant differences in protein retention were observed among diets D1, D2, and D3 ($p>0.05$), though D1 retained more protein than D2 and D3 (Figure 4A). Protein retention varied significantly over time ($p<0.05$), following a decreasing trend (Figure 4B). Group D1 decreased from 96.40 ± 0.61% at 10 minutes to 89.00 ± 0.80% at 40 minutes (7.40% leaching), group D2 from 97.45 ± 0.49% to 87.60 ± 0.11% (9.85% leaching), and group D3 from 98.01 ± 0.72% to 86.61 ± 0.59% (11.40% leaching) (Figure 4C).

Table 2 also indicates significant effects of both time ($F(3, 24) = 156.636$, $p<0.001$) and diet group ($F(2, 24) = 29.459$, $p<0.001$) on lipid retention percentages. The interaction between diet group and time is not significant ($F(6, 24) = 1.948$, $p = 0.114$), suggesting a consistent impact of diet across different time intervals. Lipid retention significantly differs among diets D1, D2, and D3 ($p<0.05$), following the trend D1 > D2 > D3 (Figure 5A). Lipid retention ratios also significantly vary over time ($p<0.05$) (Fig. 5B), showing a steady decline. The lipid retention by D1 decreased from 97.38 ± 0.41% at 10 minutes to 90.21 ± 1.36% at 40 minutes (7.17% leaching). D2 decreased from 96.42 ± 0.12% to 88.49 ± 1.78% (7.93% leaching), and D3 from 95.82 ± 0.37% to 84.47 ± 0.18% (11.35% leaching) (Figure 5C).

Table 2 highlights the significant differences in water stability results between diets and over time. Higher water stability indicates less dry matter loss, suggesting greater feed cohesion, while lower stability implies greater disintegration or leaching (Lal et al., 2023). Diet D1 (5% CMC & 0% BM) had higher water stability than D2 (2% CMC & 3% BM) and D3 (0% CMC & 5% BM), indicating that dry matter retention decreased as CMC decreased and/or BM increased. D2 exhibited moderate stability when both CMC and

BM were included (Figure 3A). Water stability decreased over time from 10 to 40 minutes (Figure 3B), with no significant interaction between diet and time. Trends in nutrient retention mirrored water stability, with higher retention in D1, followed by D2 and D3 (Figure 4A and 4B). Higher water stability correlated with lower nutrient leaching (Guo et al., 2021), while lower stability led to increased leaching due to faster feed disintegration (Ighwela et al., 2013). CMC enhances feed stability by improving water absorption and forming strong inter-particle bonds, leading to better stability and higher nutrient retention over longer periods (Ayoola, 2020; Fabbrocini et al., 2012). Higher polymerisation and lower substitution rates increase its solubility, contributing to better feed stability. However, BM-based diets showed relatively poor water stability and higher nutrient leaching compared to the control with only CMC addition. This is due to their composition, lacking CMC's strong binding properties in D2 or total binding properties in D3, which, along with increased buoyancy, leads to higher water exposure and nutrient leaching. The nature of yeast and baking powder, components of BM, disrupts CMC's binding properties or dilutes its effectiveness (Abubakar et al., 2016; Solomon et al., 2011). Their higher water absorption enhances buoyancy but challenges water stability, promoting further nutrient loss (Onada & Ogunola, 2019).

CMC produces varied effects on aquafeed performance compared with traditional binders (Adebayo et al., 2003). Studies using CMC reported water stability ranging from 25 minutes to 24 hours (Brown et al., 2015). For example, one study noted 97.53% pellet integrity at 30 minutes, and others documented improvements in pellet durability and water stability for trials lasting up to 3 hours (Aksoy et al., 2022). In contrast to this, other research trials have found that CMC can yield poorer stability relative to corn starch, wheat glucan, or α-starch. For example, in a study conducted by Ayuhafizah et al. (2024) on *M. rosenbergii* larvae and postlarvae, CMC improved pellet stability, but it was less effective than other binders like agar in promoting growth performance. Concentration-dependent effects using CMC have also been reported with optimal water stability and dry matter retention, with an inclusion level of 2-3% CMC. Higher concentrations of up to 14% were associated with poorer feed conversion and reduced lipid content (Brown et al., 2015), suggesting a potential negative impact of excessive CMC on nutrient utilisation. Consistent with this, Yusoff et al. (2021) demonstrated that pellets containing 2% and 3% CMC showed minimal disintegration and good water stability after immersion for 120 minutes. These findings indicate that CMC can enhance aquafeed stability and pellet quality under certain conditions, though its effects on water absorption and nutrient retention are influenced by species, diet formulation, and inclusion rate.

The water stability of the diets ranged from 68.61% -70.80%, the percentage of protein retained ranged from 86.61%-89.01%, and the percentage of lipid retained ranged from 84.47%-90.21%. The greatest loss of dry matter occurred during the first 0-10 minute

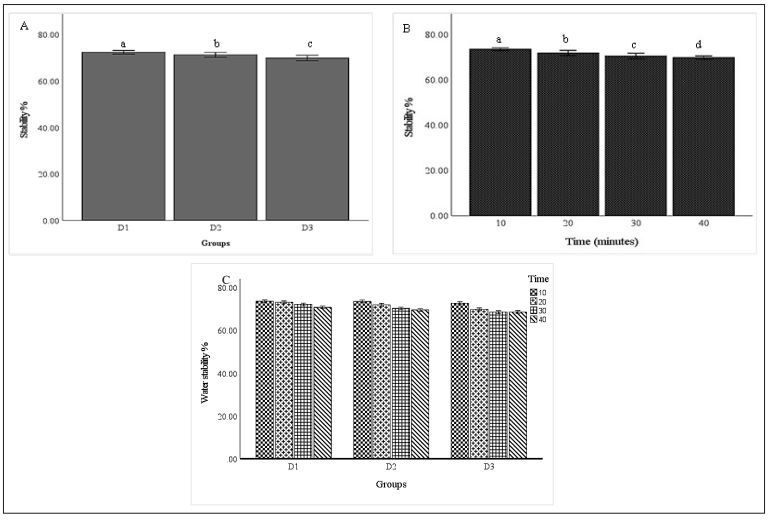

Figure 3. Water stability of the three experimental egg custard diets; A. Mean water stability for each dietary group: D1 = 5% CMC & 0% BM, D2 = 2% CMC & 3% BM, and D3 = 0% CMC & 5% BM, across the 40-minute immersion assessment; B. The mean water stability averaged across all three dietary groups showed the gradual decline in water stability in 10-minute intervals over time the 40-minute assessment period; C. Water stability of each diet following immersion after 10, 20, 30 and 40 minutes. Error bars represent the standard deviation (three replicates). Different superscript letters show significant differences in stability at $p \leq 0.05$

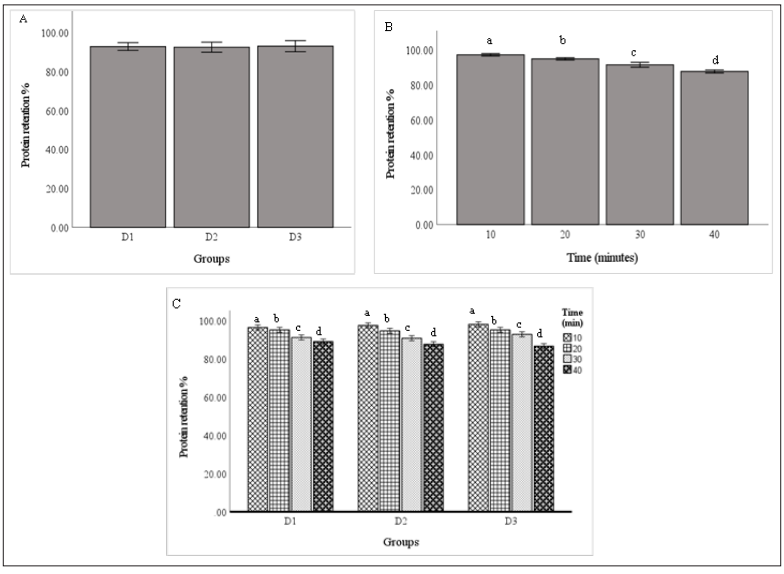

Figure 4. Protein retention rate of the experimental egg custard diets; A. Average protein retention rate for each diet across the entire 40-minute immersion period, where D1 = 5% CMC & 0% BM, D2 = 2% CMC & 3% BM, and D3 = 0% CMC & 5% BM; B. Average protein retention, averaged across all three feed diets, at 10-minute intervals for up to 40 minutes; C. The percentage of protein retained in each diet at each time point (10, 20, 30, and 40 minutes). Error bars represent standard deviations of three replicates; different superscript letters indicate significant differences at $p \leq 0.05$

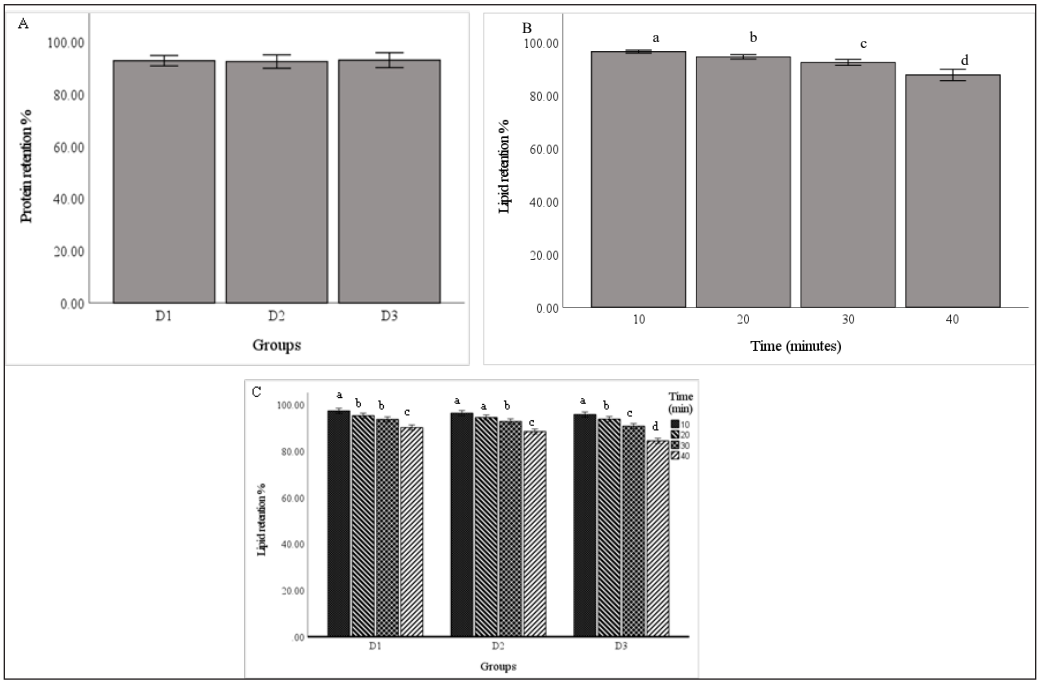

Figure 5. Performance of lipid retention displayed by the different feed groups; A. Differences in lipid retention between the three groups: D1 control: 5% CMC & 0% BM), D2 (2% CMC & 3% BM), and D3 (0% CMC & 5% BM); B. Average lipid retention, averaged across all three feed diets, at 10-minute intervals for up to 40 minutes; C. The percentage of lipid retained by each feed group after immersion in water for 10, 20, 30, and 40 minutes. Error bars represent standard deviations; superscript letters indicate significant differences at $p \leq 0.05$

interval, a period when initial dissolution is most rapid. CMC most likely achieves initial stabilisation by binding ingredients together and forming a protective barrier against water penetration, thus slowing the rate of nutrient leaching. However, prolonged exposure allows water to gradually penetrate the CMC matrix, eventually dissolving and carrying away bound nutrients.

Larval Performance

The study demonstrates the superior performance of diet D2 (2% CMC & 3% BM), followed by diet D3 (0% CMC & 5% BM), compared to the control diet (D1) (Table 3). Diet D2 consistently produced the best results. The control group (D1) exhibited the lowest survival rate at 53.3 ± 3.35%, while the highest survival rates were observed in groups fed diets D2 (66.00 ± 0.54%) and D3 (63.78 ± 2.80%), both of which were significantly higher than the control ($p<0.05$). In terms of dry weight (DW) of PL, diet D2 (1.81 ± 0.10 mg) resulted in significantly higher DW ($p<0.05$) compared to the control group D1 at

1.50 ± 0.10. The DW of larvae fed on D3 (1.66 ± 0.20 mg) showed no statistically significant differences ($p \geq 0.05$) from those fed on D1 and D2. The mean total length (TL) of the PL at the end of the feeding trial ranged from 8.61 to 9.3 mm, with groups fed D2 and D3 showing no significant difference in TL ($p > 0.05$), but both were significantly greater than the control group ($p < 0.05$). Different dietary treatments significantly influenced production and metamorphosis rates ($p < 0.05$). The D2 and D3 treatments had high levels of final mean production (larvae and PL production), which were 39.6 ± 0.33/L and 38.26 ± 1.37/L, respectively, both significantly higher than the control treatment at 31.86 ± 1.64/L ($p < 0.05$). Similarly, the metamorphosis rates in larvae fed D2 and D3 were 62.7 ± 0.51% and 60.58 ± 2.17%, respectively, which did not differ significantly from each other but were considerably higher than those in the control treatment at 50.45 ± 2.60%.

Buoyant feeds play a crucial role in larval crustacean feeding behaviour and nutrient absorption, as feeds that remain suspended in the water column are more accessible to larvae, leading to improved feed consumption, growth, and survival (Langdon & Barrows, 2011). In the present study, the water stability and nutrient leaching outcomes of D2 and D3 were lower than those of D1; however, the *M. rosenbergii* larvae fed on the D2 (3% BM & 2% CMC) and D3 (5% BM & 0% CMC) diets, displayed significantly superior survival and growth performance compared to the control group (5% CMC; 0% BM) ($p < 0.05$). This suggests that incorporating BM into diets effectively maintains feed buoyancy, improving growth outcomes by ensuring larvae can consume sufficient quantities before the feed sinks. The higher BM dosage in D3, without CMC binder, significantly outperformed the control group (D1), though it did not result in further enhancement compared to D2. This suggests that beyond 3% BM, additional performance gains may be limited by nutrient availability rather than buoyancy alone. Therefore, the incorporation of both a binder and a floating agent is a preferable choice.

Table 3
The larviculture performance of Macrobrachium rosenbergii *when fed the three experimental feeds that incorporate different inclusions of CMC and BM*

Performances	5% CMC, 0% BM	2% CMC, 3% BM	0% CMC, 5% BM
	D1	D2	D3
Mean survival (%)	53.11 ± 3.35 ^{b}	66 ± 0.54 ^{a}	63.78 ± 2.8 ^{a}
Body length (mm)	8.61 ± 0.16 ^{b}	9.4 ± 0.20 ^{a}	9.3 ± 0.10 ^{a}
Dry weight (mg)	1.50 ± 0.10 ^{c}	1.81 ± 0.10 ^{a}	1.66 ± 0.20 ^{abc}
Production (larvae + PL/L)	31.86 ± 1.64 ^{b}	39.6 ± 0.33 ^{a}	38.26 ± 1.37 ^{a}
Metamorphosis rate (%)	50.45 ± 2.60 ^{b}	62.7 ± 0.51 ^{a}	60.58 ± 2.17 ^{a}

Note. The values are shown as the mean ± standard deviation resulting from three replicates. Values within the same row with different superscript letters are significantly different ($p < 0.05$). *Abbreviation: BM = buoyancy modifiers; CMC = carboxymethyl cellulose

Several floating agents and binders provide nutrients (Sørensen, 2012). Effective binders contribute to pellet integrity and reduce nutrient leaching, thereby increasing nutrient availability and digestibility (Karim et al., 2022). CMC is a beneficial additive in aquaculture feeds; it acts as a binder, reducing nutrient leaching and disintegration of the pellets, thereby ensuring greater nutrient retention (Ergun et al., 2016; Yusoff et al., 2021). This leads to more efficient utilisation of proteins and dextrins, improved bioavailability, and ultimately, better feed conversion ratios and growth rates (Mohanta, 2024). While effective, CMC works best when combined with other essential feed additives like vitamins and minerals to create a comprehensive approach to sustainable and healthy aquaculture practices (Brum et al., 2025). In a study on spiny lobsters (*Jasus edwardsii*), CMC a highly digestible carbohydrate source was found to enhance nutrient absorption and growth performance in juveniles. Its use in formulated diets, both as a carbohydrate source and as a binder, can significantly improve digestibility and energy utilisation, leading to better growth outcomes (Simon, 2009a, b). It is important to note, however, that these findings are specific to spiny lobsters and may not apply to other species. In contrast to this, a study on largemouth bass (*Micropterus nigricans*) found that dietary CMC negatively impacted growth, nutrient digestibility, and gut morphology, with higher viscosity CMC showing more pronounced adverse effects (Liu et al., 2022). In a study on *M. rosenbergii* larvae and postlarvae, CMC was tested as a binder in microbound diets. The results showed that while CMC improved pellet stability, it was less effective compared to other binders like agar in promoting growth performance. Larvae-fed diets with agar as a binder reached a more advanced developmental stage (Stage XII) when compared to those fed CMC-based diets (Ayuhafizah et al., 2024).

Yeasts and their derivatives, such as brewer's and baker's yeast, offer substantial benefits in aquaculture. Yeasts are rich in proteins, carbohydrates, and bioactive compounds like β-glucans, which can enhance growth, survival, and feed efficiency across various species (Álvarez-Sánchez et al., 2018; Chotikachinda et al., 2008). Several studies have demonstrated that yeast supplements can effectively replace fishmeal and can represent a cost-efficient alternative without compromising growth performance (Huang et al., 2024; Pongpet et al., 2016). Additionally, the probiotic and immunostimulant properties of yeasts can help improve disease resistance and nutrient utilisation (Cai et al., 2023; Nhi et al., 2018; Zhang et al., 2018). Combining CMC and BM into a larval diet for *M. rosenbergii* aligns with their behavioural preferences for readily accessible and nutritionally stable food sources, leading to superior larval performance. The cost of diet production is another important consideration; while both CMC and BM are relatively inexpensive ingredients, further research is needed to determine the practical limits of BM inclusion and its potential impacts on palatability, water quality, nutrient density, and overall cost-effectiveness in relation to larval performance.

CONCLUSION

This study highlights that while the inclusion of CMC improves water stability and reduces nutrient leaching, it alone does not ensure optimal buoyancy for *M. rosenbergii* larvae. The control diet with 5% CMC had the best water stability and nutrient retention; however, this resulted in poor growth. Adding BM to the feed reduced nutrient retention and water stability but helped enhance buoyancy. By comparison, the diets with 3% BM and 2% CMC (D2) and 5% BM and 0% CMC (D3) showed superior growth and survival. From the experimental dietary formulations considered here, using 3% BM with 2% CMC resulted in maximal growth and survival of the *M. rosenbergii* larvae. The study concludes that customising the inclusions of binder and buoyancy modifier according to specific needs can help enhance larval performance.

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CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Riyad Taguemount	:	Conceptualisation, investigation, formal analysis, writing the original draft. Jarunan Pratoomyot: Supervision, formal analysis, writing - review and editing.
Andrew P Shinn	:	Supervision, writing - review and editing.
Rasina Rasid	:	Conceptualisation, supervision, project administration, funding acquisition, formal analysis, writing - review and editing.

DATA AVAILABILITY

All data presented within this article is available on request.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest or personal relationships that could have appeared to influence the work reported in this paper.

DECLARATION OF GENERATIVE AI USE

During the final revision of this manuscript, the authors used Claude.ai. to improve the text for clarity and to correct any grammatical errors. Following use of the tool, the authors take full responsibility for the content of the published article.

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Review Article

A Review of Key Factors Influencing Forest Fire and Wildfire Susceptibility: The Role of Climatic, Environmental, Topographic, and Anthropogenic Variables

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ABSTRACT

Forest fires represent a significant environmental and socio-economic challenge, driven by the complex interplay of climatic, topographic, and anthropogenic factors. Many studies have utilised a wide range of fire-influencing variables, such as temperature, precipitation, wind speed, elevation, and others, to model fire susceptibility maps and develop predictive models. However, these studies often assume that readers are already familiar with the underlying mechanisms that link these factors to fire occurrence, without providing detailed explanations of how each variable contributes to ignition probability and fire spread. This paper aims to address this gap by offering an accessible and comprehensive synthesis of key variables influencing forest fire susceptibility. Based on findings from previous systematic reviews and recent literature, 22 critical fire factors are categorised into three groups: climatic and environmental factors, topographic variables, and anthropogenic influences. For each factor, intuitive explanations of its relationship with fire occurrence are provided to guide hypothesis-driven feature selection for fire mapping and modelling. Crucially, this review emphasises that while these factors are globally prevalent, their application requires statistical validation to account for regional differences in fire regimes. By clarifying the mechanisms behind fire-influencing factors, this review supports more robust fire risk assessments and informs evidence-based strategies for wildfire prevention and management.

Keywords: Anthropogenic influences, climatic variables, fire modelling, fire susceptibility mapping, remote sensing, topographic variables, wildfire prediction, wildfire risk factors

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INTRODUCTION

Understanding forest fires is crucial for analysing the relationships between various factors and fire occurrences. With the rapid advancements in artificial intelligence and the availability of extensive global remote sensing data, numerous studies have leveraged these developments to analyse forest fire incidents, whether to understand their occurrence or to assess the extent and damage caused by these events. Despite the abundance of research, our investigation into fire incidents in Malaysia (Chew et al., 2022a, 2025a; Chew, Ooi, Pang, & Hoi, 2024; Chew, Ooi, Pang, & Lim, 2024) has revealed a significant gap in the literature—there is a lack of in-depth reviews that delve into the intuition and insights behind individual factors and their correlation with forest fire occurrences.

The closest comparable work is a systematic review published by Chicas and Østergaard Nielsen (2022). While it is a comprehensive study that reviewed factors utilised by researchers worldwide in forest fire studies, it primarily focused on the frequency of the fire influencing factor usage across various literature. Unfortunately, it did not explore the theoretical underpinnings or insights behind these factors. For example, why does a lower Normalised Difference Vegetation Index (NDVI) correlate with higher fire occurrences? Such insights are invaluable for early-career researchers and scientists to connect the dots between factors and their influence on fire events. This gap motivates our study, which seeks to bridge this disparity by providing a deeper understanding of each factor's role in influencing forest fire occurrences.

In this paper, we examine highly influential factors that are widely acknowledged to correlate with forest fire incidents. We aim to discuss how each factor generally impacts fire occurrences and provide intuitive explanations for their effects. Previous studies have predominantly highlighted key factors based on their regional importance or frequency of usage, often assuming that readers already possess expertise in the domain of forest fires. This has likely contributed to the lack of accessible discussions on the broader implications of these factors.

To address this, our study offers an accessible entry point for new researchers in the domain of forest fire studies. We aim to provide a comprehensive understanding of how these factors influence fire occurrences, focusing on 22 critical variables. It is important to highlight that the 22 factors to be reviewed in this study were selected as they are most frequently used in the global literature (Chicas & Østergaard Nielsen, 2022); however, this frequency of usage does not guarantee causal relevance in every local context. In fact, as noted by previous authors, the frequency of use is often related to data availability rather than physical necessity. Therefore, this review serves to provide an intuitive basis for these factors to guide feature selection. Consequently, further analysis and investigation should be conducted by researchers in their local context or specific study area to statistically validate these variables. Some of the techniques that can be considered include

Information Gain, Correlation Analysis, or Random Forest variable importance ranking. This is necessary to avoid circular reasoning, where factors are selected simply because they have been commonly adopted across the global literature. Additionally, researchers must also acknowledge the fundamental uncertainty and resolution limits associated with satellite-based remote sensing data, which serves as the primary source for these models. The subsequent discussion is organised into five sections: Section 2 summarises insights from previous systematic reviews, Section 3 covers climate and environmental factors, Section 4 focuses on topographic variables, Section 5 explores anthropogenic factors, and Section 6 provides the conclusion, highlighting key findings and future research directions.

INSIGHTS FROM PREVIOUS SYSTEMATIC REVIEWS OF FOREST FIRE FACTORS

Forest fires are complex events influenced by a multitude of factors. In the systematic review (Chicas & Østergaard Nielsen, 2022) an extensive exploration was undertaken to identify the most influential factors affecting forest fires. The exhaustive analysis examined a total of 144 factors from 94 publications spanning the years from 2001 to 2021. Among the factors that emerged as highly significant were slope, elevation, aspect, land cover, NDVI, temperature, precipitation, and wind speed. It is important to note that the sequence in which these factors are listed does not indicate their rankings. The frequency of factor usage among the 94 articles has been leveraged to establish their rankings. Notably, the prevalence of these factors was attributed to their wide global availability in existing databases, unlike more specific anthropogenic factors that require extraction from local databases.

While the review was comprehensive in reporting the frequency of use of each influencing factor across many studies, it implicitly assumed that readers are familiar with these factors and their correlations with fire ignition. To address this gap, this paper focuses on investigating the influence of the general key factors impacting forest fires, specifically categorising them into three groups: (i) climatic and environmental factors, (ii) topography, and (iii) anthropogenic factors. This categorisation is adopted from the literature to provide a structured framework, although we acknowledge that overlaps exist, particularly for variables like Land Cover, which operate across categories. Furthermore, 'climatic' and 'environmental' categories are combined in this paper as their distinction in fire modelling is often fluid. For instance, environmental variables such as soil moisture are predominantly driven by climatic conditions, making a strict separation impractical. Examining the role of these factors provides valuable insights into their relationship with fires, enriching the understanding of fire behaviour. Moreover, it aids in determining the factors that should be extracted, guiding the selection of relevant variables for constructing a fire susceptibility map or a fire model.

It is crucial to differentiate between fire susceptibility mapping and causal physical modelling, as the primary objectives of these modelling techniques are different. Susceptibility mapping relies on statistical correlations for predictive modelling using machine learning or statistical methods. On the other hand, causal physical modelling, which includes physical-based models and Computational Fluid Dynamics models typically adopt mathematical equations based on the fundamentals of fire behaviour, such as heat transfer or fluid dynamics, to simulate the occurrence or spread of fire. In contrast, this review focuses on the former, susceptibility mapping, by synthesising spatially explicit variables commonly utilised as the input for machine learning and statistical modelling. These intuitive explanations serve as a guide for feature engineering and data preprocessing, rather than a definitive description of physical fire dynamics in every biome.

The value of moving from purely data-driven approaches to those grounded in physical intuition is exemplified by our previous case studies. Our study focused on a specific fire incident that occurred in March 2023 in the Rompin district of Pahang, Malaysia, where the spatial extent of the burnt area was validated using the MCD64A1 dataset (Chew, Ooi, & Pang, 2023). This incident was analysed in relation to temperature and precipitation, leveraging the Google Earth Engine platform (Chew, Ooi, Pang & Lim, 2023). A ten-year temporal analysis of the location revealed that the highest temperatures and lowest precipitation levels consistently occur around March. This window aligns with the twenty-year FIRMS hotspot trends observed in the region (Chew et al., 2022b), showing that temperature increases and precipitation decreases as the primary fire month of March approaches. Such localised validation reinforces the necessity of understanding the underlying mechanisms of each factor to ensure robust feature selection for fire susceptibility models.

CLIMATE AND ENVIRONMENTAL FACTORS

Climatic and meteorological conditions significantly influence the likelihood of fire occurrence, as they directly affect moisture levels, fuel accumulation, rate of spread, and combustion dynamics (Syphard et al., 2008; Vilar et al., 2010). Factors such as precipitation, temperature, solar radiation, and wind speed play a pivotal role in the life cycles of flora and fauna, influencing fuel production, facilitating fuel drying for ignition, and affecting the spread of wildfires (Tavakkoli Piralilou et al., 2022). The key climate and environmental variables considered in this study, along with their definitions, optimal conditions for fire occurrence, and justifications, are summarised in Table 1.

While Table 1 provides an overview of individual climatic and environmental factors, a practical consideration for modellers is the issue of multicollinearity. Some of the variables listed in Table 1, including NDVI, NDMI, and soil moisture, are by nature highly correlated and physically related. Hence, multicollinearity analysis, such as the Variance Inflation Factor (VIF),

Table 1
Summary of fire influencing factors (Climate and environmental factors)

Factors	Short Definition	Optimal for Fire	Justification	References
Temperature	Degree of hotness	(+) Higher	Lower humidity Lower soil moisture Lower fuel moisture Drier condition Drier fuel (dead leaves/ branches) Drier vegetation Higher Drought Index Lower NDVI Amplifying evaporation and plant transpiration	(Ghorbanzadeh, Valizadeh Kamran, et al., 2019; Nur et al., 2023; Vardoulakis et al., 2020; Westerling et al., 2016)
Precipitation (Rainfall)	Rainfall	(-) Lower	Lower humidity Lower soil moisture Lower fuel moisture Drier condition Drier fuel (dead leaves/ branches). Drier vegetation Higher Drought Index Less heat energy to ignite fire	(Bravo et al., 2010; Eskandari et al., 2021; Nur et al., 2023)
Wind speed	Air movement	(+) Higher	Higher rate of fire spread	(Li et al., 2021; Syphard et al., 2008)
NDVI	Vegetation density	(-) Lower	Drier vegetation Reduced fuel moisture	(Bui et al., 2017, 2019)
Soil moisture	Water Content in Soil	(-) Lower	Lower soil moisture Drier vegetation Drier fuel (dead leaves/ branches) Less heat energy to ignite fire	(Bartsch et al., 2009; Chuvieco et al., 2004; Laguardia & Niemeyer, 2008; Sazib et al., 2022)

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Table 1 (*continued*)

Factors	Short Definition	Optimal for Fire	Justification	References
Palmer Severity Drought Index (PDSI)	Assessing drought conditions based on precipitation and temperature	(-) Lower	Lower PDSI Insufficient moisture in soil and atmosphere to meet the needs of the local ecosystem	(Palmer, 1965; Saim & Aly, 2022)
Solar radiance	Amount of radiant energy emitted by sun received by the earth's Surface	(+) Higher	More sunlight exposure Higher temperature Lower soil moisture Drier vegetation	(Babrauskas, 2014; Byram, 1943; Haigh, 2011; Kuznetsov & Baranovskiy, 2013; Sayad et al., 2019)
Topographic Wetness Index (TWI)	Water accumulation in relation to topography	(-) Lower	Less space for water accumulation Lower soil moisture Drier vegetation	(Adams et al., 2021; Harris & Taylor, 2015; Kane et al., 2015; Porensky et al., 2018; Pourtaghi et al., 2016; Sörensen et al., 2006)
Land Surface Temperature (LST)	Temperature of earth's surface	(+) Higher	Drier condition Drier vegetation Drier fuel	(Li et al., 2023; Maffei et al., 2018; Sekertekin & Bonafoni, 2020)
Normalised Difference Moisture Index (NDMI)	Water content in vegetation	(-) Lower	Lower water content in fuel (vegetation) Drier fuel Drier vegetation	(Gao, 1996; Jackson et al., 2004; Rabiei et al., 2022; Sakellariou et al., 2020)
Relative humidity	Moisture in air	(-) Lower	Less water is absorbed from atmosphere by vegetation Lower soil moisture	(Feistel & Lovell-Smith, 2017; Peng et al., 2006)
Land cover	Earth's landscape	Higher human activities land cover	Land surfaces with a higher potential for interaction with human elements have a greater likelihood of fire occurrence in the case of anthropogenic fires	(Bustillo Sánchez et al., 2021; Nur et al., 2022, 2023; Oliveira et al., 2012; Syphard et al., 2008; Tavakkoli Piralilou et al., 2022; Vilar et al., 2010)

can be exploited to perform the check. Furthermore, dimensionality reduction techniques such as Principal Component Analysis (PCA) can also be performed before introducing the variables into a predictive model to prevent redundancy.

Temperature

Elevated temperatures are undeniably linked to the occurrence of wildfires (Ghorbanzadeh, Valizadeh Kamran, et al., 2019), with prolonged dry seasons globally leading to unprecedented bushfire incidents (Vardoulakis et al., 2020). These temperature increases are frequently linked to drier conditions (Westerling et al., 2016), amplifying evaporation and plant transpiration, consequently reducing soil moisture levels (Pourtaghi et al., 2015). According to Nur et al. (2023), heightened temperatures significantly raise the probability of fire ignition or sustained burning by bringing the fuel closer to its ignition point, thereby accelerating the combustion process. These changes in climatic conditions significantly impact forest ecosystems, leading to vegetation dryness and increased vulnerability to forest fires. The combination of elevated temperatures and decreased soil moisture establishes an environment where forests are more susceptible to ignition and rapid fire spread.

Precipitation

Precipitation, also referred to as rainfall, significantly influences forest fire dynamics (Bravo et al., 2010). It plays a crucial role in determining moisture levels and the distribution of vegetation, thereby impacting both the moisture content of the fuel and the soil, ultimately affecting the rate at which fires spread (Eskandari et al., 2021; Nur et al., 2023).

Reduced rainfall leads to lower soil moisture content, making forests more susceptible to fire ignition and spread. With lower soil moisture levels, vegetation becomes drier and more susceptible to ignition. The accumulation of dry fuels, such as dead leaves and branches, creates an environment highly conducive to combustion. Additionally, reduced moisture in live fuels makes them more prone to ignition, requiring less heat to be ignited. Drought conditions, often resulting from low precipitation, further increase the risk of forest fires. The combination of dry fuels and decreased vegetation moisture fosters rapid fire spread and intensification.

Conversely, higher precipitation during the fire season can hinder fire occurrence by elevating fuel moisture levels, limiting ignition, and controlling fire spread (Bravo et al., 2010). However, Oliveira et al. (2012) suggested that precipitation occurring outside the fire season might influence fire incidents by stimulating seasonal vegetation growth. This growth could potentially increase the availability of fine fuels, particularly in grasslands, facilitating easier ignition and fire spread during the primary fire season (Oliveira et al., 2012).

Wind Speed

Wind speed significantly impacts the dynamics and spreads of wildfires (Li et al., 2021; Syphard et al., 2008). Strong winds play a significant role in rapidly spreading fires across the landscape and carrying the fire over long distances, igniting new spots several kilometres ahead of the primary fire front (Nur et al., 2023). Cruz and Alexander (2019) comprehensively analysed 118 wildfires in different ecosystems, including temperate shrublands, Australian dry eucalypt forests, and North American conifer forests. It was found that the forward rate of spread of fires in dry forest conditions is strongly influenced by wind speed. The study revealed that the forward rate of spread was approximately 10% of the wind speed. In other words, a higher wind speed corresponds to a higher rate of fire spread, leading to more rapid and extensive spread of fires.

Normalised Difference Vegetation Index (NDVI)

NDVI serves as an indicator of vegetation density and coverage near the Earth's surface, directly influencing the probability of forest fires (Bui et al., 2017, 2019). This index characterises the condition, health, and moisture content of vegetation (Nhongo et al., 2019). A decline in NDVI values indicates dry vegetation, signifying reduced water levels and elevating the potential for wildfires (Bui et al., 2017; Nur et al., 2023).

Soil Moisture

Soil moisture, identified as a crucial factor in plant physiological processes, serves as an indicator of drought conditions (Laguardia & Niemeyer, 2008). It significantly influences the moisture levels of live fuels, impacting the heat required for plant ignition (Bartsch et al., 2009). Furthermore, soil moisture directly governs fuel dryness, affecting dead fuels primarily found in the ground, thereby serving as an indicator of drought conditions (Chuvieco et al., 2004). This moisture content directly influences the dryness levels and water equilibrium of fuels, profoundly impacting the dead vegetation lying on the surface (Sazib et al., 2022).

Palmer Drought Severity Index (PDSI)

Drought Index, which is based on the Palmer Drought Severity Index (PDSI), is widely used to gauge agricultural drought by considering precipitation and temperature effects on soil moisture availability. It utilises a temperature-driven evapotranspiration algorithm to deplete moisture levels (Saim & Aly, 2022). This index's data provides assessments for landscape drought and surface water balance.

The PDSI, developed by Palmer (1965) uses a mathematical model to evaluate drought severity. It combines precipitation, temperature, and locally available water capacity to

quantify moisture supply and demand over time. Detailed descriptions of this model can be found in Palmer's original manuscript (Palmer, 1965). Lower PDSI values signify insufficient soil and atmospheric moisture to meet ecosystem needs, highlighting drought conditions.

Solar Radiance

Solar radiance, also known as solar radiation, refers to the total amount of radiant energy emitted by the Sun and received by the Earth. The energy necessary for life and driving various environmental processes, such as photosynthesis, evaporation, and climate patterns (Haigh, 2011). The amount of solar radiation received on Earth's surface varies significantly depending on seasonal cycles, slope orientation, and terrain gradient (Byram, 1943). For instance, north- and south-facing slopes receive differing amounts of solar radiation due to the Sun's changing position throughout the year, while steep and gentle slopes influence the intensity and distribution of sunlight received.

Elevated levels of solar radiation are closely associated with increased temperatures and decreased soil moisture, as higher radiation enhances evaporation rates, thereby exacerbating water deficits in the soil (Sayad et al., 2019). Furthermore, prolonged exposure to high solar radiation can heighten the risk of wildfires by drying vegetation and soils, creating conditions conducive to ignition and spread. In certain cases, concentrated solar rays may act as an ignition source for forest fires, further emphasising the link between solar radiation and wildfire risk (Babrauskas, 2014; Kuznetsov & Baranovskiy, 2013).

Land Surface Temperature (LST)

LST refers to the temperature of the Earth's surface as measured by remote sensing instruments, typically on satellites. It serves as an indicator of the energy and water exchange between the land surface and atmosphere, influencing the rate and timing of plant growth (Li et al., 2023). Additionally, LST affects surface energy balance, regional climates, heat fluxes, and energy exchanges (Sekertekin & Bonafoni, 2020).

LST is a crucial parameter in various environmental and climatic studies, including those related to forest fires, as it provides insight into the heat emitted from the ground, which can influence vegetation dryness and fire risk. High air temperatures and prolonged periods without rainfall may increase the LST captured by remote sensing devices (Maffei et al., 2018). Generally, higher LST signifies drier conditions that make vegetation more prone to ignition. Studies indicate that higher LST anomalies usually result in longer forest fire durations and larger affected areas (Maffei et al., 2018).

Normalised Difference Moisture Index (NDMI)

NDMI was devised by Gao (1996) to estimate the water content of vegetation. It is also used to assess water stress levels in vegetation, where water stress refers to the condition in which vegetation experiences insufficient moisture for growth. NDMI is also instrumental in agriculture for crop yield estimation and drought assessment (Jackson et al., 2004), and it also plays a crucial role in monitoring changes in plant health. NDMI provides critical insights for forest fire risk assessment by indicating moisture levels within vegetation. In forest fire susceptibility mapping, higher NDMI values correlate with lower forest fire risk due to higher detected moisture content (Sakellariou et al., 2020). Studies have also shown a high correlation between fire points and NDMI (Rabiei et al., 2022).

Relative Humidity

Relative humidity is a measure of the amount of moisture in the air compared to the maximum amount the air can hold at a given temperature (Feistel & Lovell-Smith, 2017). It plays a significant role in forest fire risk assessment, as higher relative humidity levels help keep vegetation moist by allowing them to absorb moisture from the atmosphere, thereby reducing fire risk. Conversely, lower relative humidity levels can lead to the drying of vegetation and increased fire susceptibility (Peng et al., 2006).

Land Cover

Land cover, also known as land use, represents the surface features of the Earth's landscape and has been linked to the incidence of fires (Nur et al., 2022; Syphard et al., 2008; Vilar et al., 2010). It depicts the arrangement, structure, and intrinsic characteristics of the landscape (Nur et al., 2023). Varied characteristics inherent in different land use types, such as their load and moisture content, can significantly impact the initiation and spread of fires (Bustillo Sánchez et al., 2021). In cases of anthropogenic fires, land cover signifies the vegetation within a particular area, portraying different fuel types that reflect their potential interaction with human elements (Oliveira et al., 2012). Different land cover patterns exert distinct influences on wildfire distribution and risk, a relationship closely associated with the interplay between the type of cover and human activity is also suggested by (Tavakkoli Piralilou et al., 2022).

Furthermore, in the context of remote sensing-based susceptibility mapping, land cover classes often serve as the primary proxy for fuel continuity. While fuel continuity is a critical determinant of fire spread, it is rarely treated as a standalone spatial variable due to the difficulty of directly measuring continuous fuel loads via satellite imagery.

TOPOGRAPHY

Topographic characteristics exert influence over vegetation distribution, composition, and flammability, and they play a significant role in local climate variations (Prasad et al., 2006). The structure of the forest canopy and the composition of species within it are notably affected by the variations in topography across the landscape (Kalantar et al., 2020). Variables such as elevation, slope, and aspect were acquired from the DEM to characterise topographic attributes. The primary topographic variables examined in this study, including their definitions, optimal conditions for fire occurrence, and supporting justifications, are summarised in Table 2.

Elevation (Altitude)

Elevation, also commonly known as altitude, signifies the vertical distance above a specific reference point, typically sea level, and generally represents the height of the land's surface above this reference point. Elevation serves as a critical factor influencing the spread and severity of wildfires, exhibiting associations with local climatic variations, vegetation distribution, composition, and flammability (Ghorbanzadeh, Valizadeh Kamran, et al., 2019). Additionally, it stands as a pivotal factor linked to temperature, moisture, and wind dynamics, profoundly impacting vegetation structure, fuel moisture, and air humidity (Oliveira et al., 2014). Regional climate differences are notably influenced by elevation, establishing it as a crucial feature (Tavakkoli Piralilou et al., 2022).

Research by Pu et al. (2007) found that most burned areas in North America occur at lower elevations (below 500 m). Similarly, Mohammadi et al. (2014) observed that fires are more frequent at lower elevations with gentle slopes and short distances from farmland, areas where human activity is concentrated. These findings suggest that human activities near farmland, typically situated at lower elevations, contribute to higher fire occurrence. While tourism activities such as camping (Suhardono et al., 2024) are present at both lower and higher elevations, human activity is typically more concentrated at lower elevations. This higher density of human presence at lower elevations has been speculated to contribute to elevated fire risk

Higher moisture levels found in elevated terrains act as a preventive measure against severe wildfires (Ghorbanzadeh, Blaschke, et al., 2019). Observations have indicated that fire behaviour trends are less distinct at higher altitudes due to increased precipitation levels (Ljubomir et al., 2019). However, Hong et al. (2019) speculate the possibility of widespread fire occurrence is heightened at higher altitudes due to the impact of wind action.

Overall, the literature presents mixed findings on whether higher or lower elevations are more prone to wildfire occurrence, as this relationship depends on regional factors and interacting environmental conditions. These contradictions likely stem from regional

Table 2
Summary of fire influencing factors (Topography)

Factors	Short Definition	Optimal for Fire	Justification	References
Elevation (Altitude)	Vertical distance above sea level Note: Affects climate, vegetation distribution, and composition.	(-) Lower	Higher temperature Higher accessibility More human activities	(Mohammadi et al., 2014; Pu et al., 2007; Suhardono et al., 2024)
		(+) Higher	Higher wind speed Higher spread rate	(Hong et al., 2019)
		(+) Higher	*Alternative Opinion (Lower Risk)* Higher precipitation Lower Temperature Lower fire risk	(Ghorbanzadeh, Blaschke, et al., 2019; Ljubomir et al., 2019)
Slope	Degree of steepness	(-) Lower	Higher accessibility More human activities	(Conedera et al., 2011; Widayati et al., 2010)
		(+) Higher	Higher windspeed Higher rate of spread	(Nur et al., 2022; Pourtaghi et al., 2015)
Aspect	Direction of slope	Southern	More sunlight energy Higher temperature Higher wind speed Lower soil moisture Lower humidity	(Adab et al., 2013; Prasad et al., 2008; Salavati et al., 2022; Sayad et al., 2019)
Hillshade	Intensity of sunlight depends on the terrain	(+) Higher	More sunlight exposure Higher temperature Drier vegetation	(Adaktylou et al., 2020; Iverson & Prasad, 2003; Razali et al., 2010)
Topographic Wetness Index (TWI)	Water accumulation in relation to topography	(-) Lower	Less space for water accumulation Lower soil moisture Drier vegetation	(Adams et al., 2021; Harris & Taylor, 2015; Kane et al., 2015; Porensky et al., 2018; Pourtaghi et al., 2016; Sörensen et al., 2006)

climatic variations. For example, in some regions, higher elevations are associated with increased precipitation and cooler temperatures which reduce fire risk, whereas in others, they may experience stronger winds and drier vegetation that increase susceptibility. Hence, the influence of elevation must be interpreted within the specific climatic context of the study area

Slope

Slope refers to the degree of incline or steepness of the land surface, and is generally expressed as a percentage or angle. It acts as an indicator of the rate of elevation change (Ljubomir et al., 2019) and controls both biodiversity and vegetation distribution (Tavakkoli Piralilou et al., 2022). Slope angles play a crucial role in fire behaviours.

Slope inclination greatly influences fire ignitions by restricting accessibility; typically, higher degrees of slope tend to limit accessibility (Oliveira et al., 2014). In a case study conducted in Switzerland (Conedera et al., 2011), findings revealed a higher frequency of anthropogenic fires occurring on gentler slopes, specifically those below 33^{o}, indicating a greater incidence of fire events. Furthermore, Widayati et al. (2010) also emphasised the restricted accessibility, indicating that a slope threshold of 20^{o} becomes a limiting factor for forest harvesting.

Slope influences both the speed and direction of fire spread (Kamran et al., 2014), often showing a high correlation with the formation and progression of forest fires (Bui et al., 2017). The rate of fire movement is faster on upward slopes. An increase in slope degree correlates with a faster rate and intensification of fire spread (Nur et al., 2022, 2023). Steep terrains significantly influence local wind formation, accelerating fire speed (Adab et al., 2013). Bustillo Sánchez et al. (2021) also accentuated that steeper areas facilitate rapid fire expansion, whereas less steep areas impede fire distribution.

Aspect

Aspect refers to the direction the slope is facing (Ljubomir et al., 2019), influencing the amount of solar radiation (i.e., sunshine) received through sunlight exposure (Salavati et al., 2022; Tavakkoli Piralilou et al., 2022). It describes the maximum rate of change in elevation between cells and their adjacent neighbours (Ljubomir et al., 2019; Prasad et al., 2008). This factor influences an area's climate in terms of exposure to temperature, winds, humidity, and fuel moisture.

Southern areas often experience more forest fires due to several environmental factors as vegetation tends to be drier and less dense on south-facing slopes compared to north-facing ones (Adab et al., 2013). South-facing aspects receive more direct heat from sunlight, resulting in higher temperatures, stronger winds, lower humidity, and decreased fuel moisture levels, leading to drier and less dense vegetation compared to north-facing

slopes (Adab et al., 2013; Prasad et al., 2008; Salavati et al., 2022). These conditions contribute significantly to an increased potential for fire occurrences. Typically, the risk of forest fires is higher on south-facing slopes as they tend to be warmer and drier than north-facing slopes (Sayad et al., 2019).

Conversely, north-facing slopes receive less sunlight compared to their southern counterparts, retaining more moisture and supporting healthier vegetation (Kamran et al., 2014). On the other hand, Kamran et al. (2014) highlighted that east-facing slopes receive direct sunlight earlier in the day compared to west-facing slopes.

On the other hand, north-facing slopes receive less sunlight than their south-facing counterparts (Kamran et al., 2014). Consequently, north-facing slopes tend to retain more moisture, supporting healthier vegetation. These studies (Ghorbanzadeh, Blaschke, et al., 2019; Kalantar et al., 2020; Pourtaghi et al., 2015) suggested that fire spreads faster on east-facing slopes due to increased solar radiation in mountainous areas.

Hillshade

Hillshade represents the intensity of sunlight on a terrain surface, highlighting variations in illumination caused by topographic features. Hence, it can be used to assess the solar radiation difference due to differences in angle, aspect, and position (Iverson & Prasad, 2003). It is commonly used in geographic analysis to visualise the effects of sunlight and shadow on the landscape. Fires tend to spread more rapidly on slopes and are affected by the aspect of the slope relative to the sun. This aligns with earlier discussions that south-facing slopes have a higher risk of forest fires (Adab et al., 2013; Sayad et al., 2019).

Hillshade values are derived from the angle of the terrain relative to the position of the light source. Hence, they do not correspond to any physical units like degrees or meters. Instead, they are a relative measure of light and shadow intensity, typically ranging from 0 to 255, where 0 indicates complete shadow (no illumination) and 255 indicates full illumination (maximum sunlight).

While no direct studies have been identified in the literature linking hillshade values to fire occurrence, Adaktylou et al. (Adaktylou et al., 2020) demonstrates that greater sunlight exposure increases surface temperature (Razali et al., 2010), leading to warmer and drier conditions and accelerating vegetation drying, which contributes to higher fire risk and intensity.

Topographic Wetness Index (TWI)

TWI (Beven & Kirkby, 1979), a quantitative metric designed to assess water availability in relation to topography and has been widely used in hydrological studies to understand how landscape features influence moisture distribution. The TWI evaluates soil moisture

by integrating water inflow from upslope catchment areas and downslope drainage for individual cells (Porensky et al., 2018). Commonly, TWI is utilised to quantify hydrological processes and topographic control (Sörensen et al., 2006).

TWI measures the landscape's tendency to accumulate water, with higher values indicating wetter areas and lower values indicating drier areas. As a result, places with higher TWI will have higher soil moisture, which can further reduce the probability of a fire occurrence. These regions typically exhibit a positive annual water balance, where water supply exceeds water loss through evapotranspiration, resulting in higher soil moisture levels (Adams et al., 2021).

Conversely, lower TWI values are associated with drier conditions, frequently observed in elevated locations such as ridge tops and plateaus. Studies (Adams et al., 2021) conducted in Mountain Ash forests have demonstrated a correlation between low TWI values and high wildfire intensity, indicating that reduced water availability significantly influences fire behavior. Consequently, TWI can be used to identify areas at least risk of fire during fire incidents characterised by high intensity and rapid spread. Additionally, TWI also serves as an important predictive indicator for fire incidents and risk assessment (Harris & Taylor, 2015; Kane et al., 2015; Pourtaghi et al., 2016).

ANTHROPOGENIC FACTORS

Anthropogenic refers to things caused or influenced by human activities in the environment. While the preceding factors focus on environmental influences affecting forest fire occurrences, various studies (Zhang et al., 2023) suggest that human activities might hold greater significant than natural phenomena in initiating wildfires. Spatial patterns of wildfire ignition exhibited a strong correlation with human access to natural landscapes, highlighting the proximity to urban (i.e., settlement) areas and roads as one of the most influential contributing factors. Moreover, the construction of roads or railways and fire sources generated from mobile vehicles will aggravate the risk of forest fires (Bui et al., 2019). The main anthropogenic variables influencing forest fire susceptibility, together with their definitions, optimal conditions for fire occurrence, and justifications, are summarised in Table 3.

Distance to Roads

Distance to roads is a crucial metric in understanding the accessibility to forest areas prone to fire ignition. Numerous studies (Martínez et al., 2009; Vilar et al., 2010) have identified the proximity to roads as a significant factor influencing forest fires, reflecting the extent of anthropogenic activity and human interactions within these landscapes. For instance, it can be assumed that a shorter distance to roads from the forest indicates easier accessibility and potentially more human activities.

Table 3
Summary of fire influencing factors (Anthropogenic factors)

Factors	Short Definition	Optimal for Fire	Justification	References
Distance to roads	-	(-) Shorter	Higher accessibility More human activities	(Martínez et al., 2009; Vilar et al., 2010)
Distance to urban area (settlement)	-	(-) Shorter	Higher accessibility More human activities	(Nur et al., 2023; Sachdeva et al., 2018; Tavakkoli Piralilou et al., 2022)
Distance to stream / river	Water resources	(-) Shorter	More human activities	(Pourtaghi et al., 2016)
		(+) Further	Further water sources Lower soil moisture Drier vegetation	(Nur et al., 2023)
Socio-economic variables	Unemployment rate	(+) Higher	High social tension Increase vandalism	(Martínez et al., 2009; Sebastián-López et al., 2008)
Nighttime light	Artificial light generated by human activities from remote sensing images	(+) Higher	More human activities	(Zhang et al., 2023)
Population density	Human Impact Index (HII)	(+) Higher	More human activities	(Oliveira et al., 2012; Sanderson et al., 2022)

However, a significant limitation of this variable, and anthropogenic factors in general, is the inability to distinguish between intentional ignitions and accidental fires. Distance to roads only indicates human accessibility, effectively aggregating both potential arsonists and accidental ignition sources into a single spatial metric, which limits the ability to model specific ignition behaviours.

Distance to Urban Area (Settlement)

Similar to the proximity to roads, the distance to settlements is a pivotal metric that gauges accessibility to forested regions and significantly influences the ignition of wildfire incidents (Sachdeva et al., 2018; Tavakkoli Piralilou et al., 2022). Factors such as the distance to recreational areas and roads, alongside human settlements, serve as indicators of accessibility to forested zones susceptible to wildfires, often attributed to human activities (Nur et al., 2023). Supporting this, Pham et al. (2020) identified distance from roads and settlements as the predominant variables influencing fire incidents in Pu Mat National Park, Vietnam.

Distance to Stream/ River

Distance to streams significantly impacts forest health by providing essential water resources (Ghorbanzadeh, Valizadeh Kamran, et al., 2019; Sachdeva et al., 2018). It is also closely tied to forest conditions, as rivers often serve as crucial water sources (Nur et al., 2023). These rivers frequently attract human recreational activities, and the associated human presence significantly contributes to wildfire incidents (Pourtaghi et al., 2016). Pourtaghi et al. (2016) found that distance to rivers, particularly in the range of 300-450 m, showed the highest correlation with fire occurrence. However, it can also be argued that shorter distances to streams or rivers may facilitate firefighting efforts by improving access to water sources. Although several studies have incorporated distance to rivers as a variable in fire susceptibility mapping and machine learning models, detailed investigations into its direct correlation with fire occurrence remain relatively limited.

Socio-economic Variables

The unemployment rate has been identified in studies within the European Mediterranean setting as a potential contributing factor to the fire occurrence (Martínez et al., 2009; Sebastián-López et al., 2008). However, the underlying reasons for this correlation remain ambiguous. This relationship is often interpreted either as a broad indicator of social tension, potentially leading to increased instances of vandalism in rural areas.

Nighttime Light

Nighttime light intensity data effectively capture various human activities, as highlighted by the authors' results in (Zhang et al., 2023). The authors utilised nighttime light data as a substitute for Gross Domestic Product (GDP) to fulfil the resolution requirement in their study. GDP, typically characterised by coarse spatial resolution, is replaced by nighttime light, which comprises remote sensing images with high spatiotemporal resolutions, relying on artificial light generated by human activities. Consequently, the authors postulated that this light intensity data more accurately and directly captures variations in human activities compared to GDP. In essence, their study highlighted nighttime light alongside population density, road density, and railway density as key socioeconomic indicators to outline human activities.

Population Density

Population density represents the distribution of potential causative agents, considering factors such as human activities and interactions that can contribute to forest fire ignition and spread (Oliveira et al., 2012). Higher population density often correlates with increased human activity. This increased activity can lead to a greater risk of fire incidents due to factors such as campfires, debris burning, and machinery use. To quantify the human footprint or human influence, the Human Impact Index (HII) (Sanderson et al., 2022) can be exploited to measure the extent of human impact on natural environments.

CONCLUSION AND FUTURE REMARKS

This study has reviewed and synthesised the key factors influencing forest fire susceptibility, highlighting the critical roles of climatic, environmental, topographic, and anthropogenic variables. Forest fire occurrences are shaped by the combined influence of these factors rather than by a single variable. Climatic conditions such as temperature, precipitation, wind speed, and solar radiation directly impact fuel moisture levels and fire behaviour. Topographic attributes, including slope, elevation, and aspect, affect local microclimates, vegetation patterns, and human accessibility, all of which contribute to variations in fire risk. Anthropogenic factors, such as proximity to roads and settlements, population density, and socio-economic indicators, further influence fire susceptibility through human activities and land-use practices.

Importantly, this paper has sought to bridge the gap in the literature by providing intuitive explanations of how each factor contributes to fire occurrence, aiming to support early-career researchers and practitioners in understanding these complex relationships. By offering a detailed synthesis of 22 critical variables, this study provides a foundation for selecting appropriate factors in fire susceptibility mapping and model development.

Nevertheless, while this review provides a general overview of global trends, local adaptation is still essential. For instance, in tropical countries such as Malaysia, where fire regimes are predominantly anthropogenic (Chew et al., 2022a) and most of the serious fires occur in peat swamp areas (Miettinen et al., 2017). These areas may be influenced by specific factors such as peat soil moisture or monsoonal rainfall patterns that differ from the standard natural fire regimes often cited in the literature. Given that various locations experiencing forest fires may be affected by distinct factors (Pourtaghi et al., 2016), it is crucial to conduct investigations that are specific to the area of interest to identify the predominant influencing factors. Furthermore, it is essential to address the temporal mismatch often found between static raster layers and dynamic fire events. To mitigate this issue, particularly in regions with distinct seasonal variations (e.g., temperate zones), we recommend utilising seasonal aggregates rather than static annual averages. This approach ensures the accurate capture of phenological changes in vegetation and varying fire risk. Future research should also aim to distinguish more clearly between ignition drivers (predominantly anthropogenic) and spread drivers (climatic/topographic), and move beyond binary occurrence mapping to assessing fire intensity and severity, acknowledging that even in human-dominated regimes, climatic conditions may act as critical amplifiers of fire intensity and severity.

To improve the accuracy of fire susceptibility model, there is also a need to integrate dynamic and high-resolution data sources, incorporating both antecedent meteorological conditions (to capture lag effects) and near real-time environmental indicators, to improve the accuracy of fire susceptibility models. Early exploratory work on temporal sampling for fire classification (Chew et al., 2020) and recent performance comparisons of machine learning algorithms across Peninsular Malaysia (Chew et al., 2025b) underscore the potential for high-fidelity detection models in this region. The application of advanced artificial intelligence and machine learning techniques offers significant potential for capturing the complex and non-linear interactions among these variables. Furthermore, interdisciplinary collaboration that combines environmental science, remote sensing, and socio-economic analysis will be essential for developing effective fire management strategies.

Ultimately, by clarifying the physical and environmental mechanisms behind these variables, this review empowers researchers to transition from purely data-driven 'black-box' approaches to hypothesis-driven modelling. This ensures that feature selection is not merely an exercise in maximising statistical metrics, but is grounded in physical reality, leading to more robust and interpretable fire susceptibility models. For instance, in the context of machine learning, researchers utilising decision trees can apply this domain knowledge to better interpret the resulting decision rules, ensuring that the model outputs are logically sound and translate into effective prevention strategies.

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AUTHOR CONTRIBUTIONS

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Morphological Differences of Experimental Hybrids and Check Varieties of High-yielding Grain Maize

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ABSTRACT

Malaysia's poultry industry heavily relies on imported grain maize, posing challenges to national food security and economic sustainability. To address this, the development of high-yielding maize varieties is critical. This study evaluated morphological and yield-related traits of ten maize genotypes, which were five experimental hybrids (GH, GJ, HI, HJ, JK) and five check varieties (BTL 1, Pacific 338, Suwan 5, TVDC, GWG888) using a Randomised Complete Block Design (RCBD) with four replications at Universiti Putra Malaysia (UPM), Selangor. Significant differences were observed across all measured parameters. Experimental hybrids, particularly GJ, outperformed check varieties in key yield determinants, including dehusked ear weight, ear diameter, and kernel row count. The most distinctive finding was that hybrid GJ achieved the highest grain yield of 11,794 kg/ha, substantially exceeding that of the check varieties. Strong positive correlations were identified between dehusked ear weight and grain weight ($r = 0.85$, p-value = 0.02, $\alpha = 0.05$), and between plant and ear height ($r = 0.78$, p-value = 0.05, $\alpha = 0.05$), indicating synchronised plant development. Importantly, dehusked ear diameter emerged as a key yield predictor, showing moderate but meaningful correlations with grain yield, kernel rows, and ear weight ($r = 0.61$ - 0.68, p-value = 0.05, $\alpha = 0.05$). These results highlight the pivotal role of ear diameter as a selection criterion in maize breeding programs. The superior performance of hybrid GJ presents a promising solution to Malaysia's maize yield gap. Adoption and further development of such high-performing genotypes could reduce import dependency, strengthen local feed grain supply chains, and enhance national food security.

Keywords: Breeding, genetics, genotype, kernel, plant traits, yield

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INTRODUCTION

Zea mays L., commonly known as maize, exhibits distinct morphology that contributes to poultry nutrition. The morphology of grain maize is characterised by a central cob, surrounded by tightly packed rows of kernels. These kernels serve as a crucial source of calories for poultry, providing the high energy levels required for their growth and development. Grain maize has been extensively used in the formulation of poultry feed, playing a vital role in creating balanced diets that meet its nutritional needs.

The demand for grain maize as poultry feed is substantial both globally and in Malaysia. In the 2021/22 period, worldwide maize production exceeded 1.2 billion metric tons, surpassing other grains, while wheat production amounted to approximately 778.6 million metric tons (Shahbandeh, 2023). However, despite this increasing demand, there is uncertainty regarding whether the future supply of maize will be able to meet the ever-increasing global demand (Nordin et al., 2018). With the increasing demand for grain maize in the poultry feed sector, Malaysia has witnessed substantial growth in the industry. Consequently, there has been a surge in the demand for grain maize within the country. To meet this demand, Malaysia relies heavily on imported grain maize. According to Wahab (2023), approximately 90% of the imported grain maize into Malaysia originates from countries such as Argentina, Brazil, and India. This heavy reliance is driven by a decrease in local grain maize production by 16% in 2021 from 2020 (Wahab, 2023). To address this challenge, it is crucial to focus on enhancing local production and improving crop yields in Malaysia. This effort is essential for ensuring a sustainable supply of grain maize for poultry feeding and reducing dependence on imports.

In the pursuit of improving crop yield, maize with desirable morphology or traits plays a pivotal role in achieving high productivity. Breeders can effectively influence grain yield by targeting specific traits such as plant height, ear height, ear weight, number of kernels per row, ear length, and others (Mohammedali et al., 2021; Mousa et al., 2021; Uba et al., 2018). It has been found that maize genotypes with shorter plant height have been found to outperform in yield compared to the taller plants (Nordin et al., 2018). However, another finding by Greveniotis et al. (2019) and Uba et al. (2018) stated that yield is primarily focused on ear traits. In particular, Haddadi et al. (2012) also observed a significant positive correlation between the number of kernels per row and grain yield. Similarly, Singh et al. (2020) identified multiple traits, including plant height, ear height, ear length, ear diameter, and the number of kernel rows per ear, that exhibited a significant positive correlation with grain yield. Understanding and selecting maize varieties with favourable traits are crucial for researchers to enhance crop performance, leading to higher yields. Consequently, developing maize varieties with improved traits has become a primary objective in most breeding programs, enabling the production of maize varieties that significantly augment yield production.

Research Objectives

The primary objective of this study is to identify characteristics among several genotypes of maize, determine the differences and similarities in traits associated with high yield, and figure out the relationships existing among these traits.

MATERIALS AND METHODS

Study Overview

The study was conducted at Field 15, Universiti Putra Malaysia, Selangor. The experimental site is located in a tropical climate region characterised by warm temperatures and frequent rainfall throughout the year.

Experimental Design

The study evaluated ten maize hybrids, consisting of five experimental hybrids and five established check varieties. The experimental hybrids included CML331 x CML383 (GH), CML331 x CML491 (GJ), CML383 x CML428 (HI), CML383 x CML491 (HJ) and CML491 x CML498 (JK). In addition, five established check varieties were included for comparison, namely Breeder Test Line 1 (BTL 1), Pacific 338, Suwan 5, TVDC and GWG888. This diverse selection of hybrids and varieties forms the basis of this investigation into various morphological traits and yield parameters.

The experiment used a Randomised Complete Block Design (RCBD) with a single factor, which was the maize genotype. The experimental layout consisted of four blocks, with each block containing ten plots corresponding to the ten genotypes, with sizes for each being 19.5 m^2 per block. Thus, the total number of experimental units was 40, giving a sample size of n = 40.

Cultural Practices

Every single hole was filled with two seeds, and thinning was done between 7 - 14 days after sowing. On days 7 and 21, the fertiliser was applied at 50 kg/ha of N, 20 kg/ha of P and 20 kg/ha of K. On day 35, the fertiliser was applied at 50 kg/ha of N, 50 kg/ha of P and 70 kg/ha of K.

Statistical Analysis

Initially, the Anderson-Darling normality test was performed to evaluate whether the data followed a normal distribution, allowing for the identification of parametric and non-parametric data. Since these were normally distributed data, the analyses were conducted using Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) as mean comparison, which were applied to the parameter with a total of ten samples when

the F-value showed a significant difference at 95% confidence level, when compared with F-value references in the F-table. In order to study the correlations between the traits, Pearson's correlation analysis was applied to measure the strength and direction of the linear relationship between two variables. All of these analyses were run using R Studio (Version 3).

Data Collection

The data collection was taken based on yield and yield components. The yield data were collected from a harvested area of 11 m^2, representing the harvested portion. In contrast, the data on yield components were acquired through a random sampling process, where a total of 10 samples were taken from the harvested area. The data were taken at a maturity level of 110 - 120 days.

A total of nine parameters were considered to evaluate the yield and other characteristics for yield components, namely plant height, ear height, dehusked ear weight, dehusked ear diameter, dehusked ear length, number of kernel rows per ear, number of kernels per row, grain weight and grain yield.

Plant Height and Ear Height

In this study, plant height and ear height were measured to assess the morphological characteristics of maize plants. Plant height was measured using a measuring tape from the ground level, specifically from the point where the uppermost brace roots emerge, to the base of the tassel on the plant, where the flag leaf is located. Similarly, ear height was measured from the ground level, starting from the uppermost brace roots' emergence to the base of the first ear on the plant.

The measurements of both plant height and ear height were recorded in centimetres (cm). The data collected was then presented as the average of plant height per plant and ear height per plant. These measurements were taken during the harvesting time, indicating that the plants were assessed at their mature stage when the ears had fully developed.

Dehusked Ear Weight

The samples were weighed using a CAMRY electronic weighing balance (Model EK3211, China). The measurements were recorded in grams (g) and were presented in average dehusked ear weight per ear.

Dehusked Ear Diameter and Length

The diameter measurements were taken using an electronic digital vernier calliper capable of measuring from 0.1 millimetre (mm) up to 150 mm. The measurements were recorded in

mm. The diameter measurements were taken on three different sections of the ears, which were the top, middle and bottom of the ear. Subsequently, the data from these sections were averaged to determine the diameter value. The data were presented in average dehusked ear diameter per ear.

On the other hand, the dehusked ear length was determined by measuring the length of the ear where kernels were produced. A ruler with a length of 30 cm was used for measuring the dehusked ear length. The data were recorded in cm and presented in average dehusked ear length per ear.

Number of Kernel Rows per Ear and Kernels per Row

The number of kernel rows per ear was determined by manually counting each dehusked ear. The data was presented in an average number of kernel rows per ear. Similarly, the number of kernels per row was manually counted on each dehusked ear. A total of three rows were randomly selected on the dehusked ear, and the number of kernels in each of those rows was counted. The counts from the three rows were averaged to calculate the average number of kernels per row per ear.

Grain Weight and Yield

In order to measure grain weight and yield, moisture content measurements were taken along during the weighing process and recorded as a percentage (%). The moisture content data collection was conducted using a digital grain moisture meter (AR991, China). The moisture content value recorded was utilised for the calculation of grain weight and yield, ensuring standardisation to a moisture content level of 15%. This standardised moisture content level holds significance as indicated by Nordin et al. (2018), who concluded that maintaining a moisture content of 15% is crucial for optimising both grain yield and quality.

The dehusked ears were manually shelled to separate the grain from the cob. The grain samples were then individually weighed to determine the grain weight per ear. Additionally, the grain from the harvested area was weighed to obtain the grain yield. The samples were weighed using a CAMRY electronic weighing balance (Model EK3211, China).

The grain weight data were presented as the average grain weight per ear and expressed in g unit. The data for grain yield were presented as the average of grain weight per plot and converted to kilograms per hectare (kg/ha).

RESULTS AND DISCUSSION

Plant Height and Ear Height

Plant height is one of the parameters that correlated with lodging resistance and yield production in maize (Wang et al., 2019). Table 1 reveals an intriguing pattern when it comes

to plant height in experimental hybrids. It is evident that the experimental hybrids differ significantly from one another, resulting in a distinct plant height for each. GH and HJ hybrids are the most distinctive, having the shortest plant height, 165.2 cm and 184.9 cm, respectively. In another study, a local maize hybrid (Sweet King) planted at Terengganu, Malaysia, recorded a plant height of 151.33 cm ± 3.20 (Nordin et al., 2018).

However, in an unexpected turn, the check variety showed no significant difference in plant height, ranging from 192.2 cm to 212.4 cm, respectively. Comparing the results with other studies, the GWG888 (192.2 cm) check variety in this study exhibits similarities with the findings from other experiments conducted at different locations in the Peninsular Malaysia area in 2019 and 2020, which recorded 197.5 cm for GWG888 (Adham et al., 2022). It is interesting to see how the performance of this revised check variety aligns with previous observations, suggesting a certain level of consistency and reliability in its growth characteristics.

Interestingly, based on the previously mentioned result, Nordin et al. (2018) categorised shorter plants as preferred genotypes because of their possible influence on root attachment to the soil. This evidence supports the notion that shorter plant heights may be advantageous, especially in withstanding strong winds and preventing toppling.

In addition to plant height, ear height also showed significant differences between experimental hybrids and the check varieties (Table 1) (p-value = 0.003). The ear height of Pacific 338, BTL 1, HI, JK, Suwan 5 and GH ranged from 98.8 cm to 110.5 cm, and were significantly different from GH, GWG888, TVDC and HJ (81.2 cm to 91.9 cm). The difference in ear height between these two groups was 16.77%. In other studies, it has been suggested that an ear height of 78.5 cm is considered acceptable and desirable (Nordin et al., 2018). It is because shorter ear heights will lead to issues of a crowded canopy, poor aeration and limited sunlight transmission to lower sections that can lead to a significant decrease in crop output (Nordin et al., 2018).

Moreover, another study recorded that crosses of M 703 x M 733 achieved better yielding with an ear height of 101.7 cm compared to other crosses with higher ear heights (El-Hosary et al., 2018). These findings indicate a general preference for higher ear heights, as they facilitate better crop yield by allowing proper canopy spacing, improved aeration, and optimal sunlight penetration. However, it is important to note that excessively high ear placement can lead to lodging and have a negative impact on the final yield (Nordin et al., 2018).

Dehusked Ear Weight

The dehusked ear weight is a key factor in determining the yield of maize, as a higher weight indicates that the maize plant has produced more kernels. The dehusked ear weight (Table 2) of maize hybrids varied significantly (p-value = 0.000003), with JK, HJ and GJ

Table 1
The effects of genotypes on selected experimental and check varieties on plant height and ear height of grain maize

Genotype	Plant Height/Plant, cm	Ear Height/Plant, cm
GH	165.2±12.6c	81.2±4.8c
GJ	209.9±17.9a	110.5±13.3a
HI	200.1±6.2ab	100.8±1.8ab
HJ	184.9±29.5bc	91.9±22.3bc
JK	200.9±13.5ab	103.7±9.1ab
BTL 1	193.7±24.3ab	99.2±8.4ab
Pacific 338	211.7±14.9a	98.8±7.2ab
Suwan 5	209.6±12.0a	108.4±7.0a
TVDC	212.4±11.9a	88.6±10.1bc
GWG888	192.2±11.4ab	83.1±7.0c
cv	7.460	10.143

Note. Means followed by the same letter are not significantly different among genotypes by the DMRT test at p-value ≤ 0.05. Values are presented as mean ± standard error (SE). CV refers to the coefficient of variation, which indicates the relative variability of the data

Table 2
The effects of genotypes on selected experimental and check varieties on dehusked ear weight, dehusked ear diameter, dehusked ear length, kernel rows per ear and kernels per row of grain maize

Genotype	Dehusked Ear Weight/Ear, g	Dehusked Ear Diameter/Ear, mm	Dehusked Ear Length/Ear, cm	Kernel Rows per Ear	Kernels per Row
GH	246.1±18.9bc	46.24±0.79bc	17.29±0.30cd	12.4±0.3e	36.6±1.0bcde
GJ	298.9±25.7a	50.30±0.63a	16.55±0.89cd	14.8±0.3b	38.1±1.7abc
HI	206.1±24.0d	42.12±2.22d	16.35±0.97cd	12.5±0.2e	35.0±2.1cdef
HJ	289.6±14.5a	46.63±0.49b	18.97±1.75ab	14.0±0.1bcd	40.2±2.7a
JK	273.2±11.6ab	49.76±0.89a	16.26±0.87d	15.6±0.7a	33.7±1.6ef
BTL 1	253.3±23.9bc	47.11±1.07b	17.13±1.03cd	14.3±0.6bc	34.4±2.2def
Pacific 338	256.7±14.0bc	46.81±1.0b	17.08±0.344cd	13.7±0.4cd	37.3±0.9abcd
Suwan 5	226.7±19.0cd	45.40±1.94bc	17.83±1.02bc	13.4±0.5d	34.5±2.6def
TVDC	253.5±23.9bc	44.54±2.10c	17.44±0.29cd	13.6±0.7cd	32.0±2.3f
GWG888	256.7±10.7bc	45.25±0.38bc	19.63±0.38a	14.7±0.4bc	38.3±1.0ab
cv	7.649	2.729	5.376	3.644	5.319

Note. Means followed by the same letter are not significantly different among genotypes by the DMRT test at p-value ≤ 0.05. Values are presented as mean ± standard error (SE). CV refers to the coefficient of variation, which indicates the relative variability of the data

exhibiting the highest weights of 272.2 g, 289.6 g and 298.9 g, respectively. In contrast, HI and Suwan 5 had the lowest recorded weights of 206.1 g and 226.7 g, respectively. This represents a 9% lower from the genotype with the highest dehusked ear weight. In a study conducted by El-Hosary et al. (2018), it was shown that the crossbreeding of M 702 x M 722 resulted in an ear weight of 249.2 g, leading to a significant increase in yield. Conversely, the crossbreeding of M 702 with M 704 resulted in a lower ear weight of 161.0 g (El-Hosary et al., 2018).

This variation in dehusked ear weight among the hybrids can be attributed to several factors, including genetic traits, environmental conditions, soil fertility, and agronomic practices employed during cultivation. As the environmental conditions in this study were standard for all hybrids, this parameter might be influenced by the genetic traits. More than 70% of ear attributes have heritability values over 0.5, suggesting that genetic factors continue to have a significant impact on maize ear features (Wang et al., 2023). In another finding, a total of four single-nucleotide polymorphisms (SNPs) were identified, with two SNPs associated with both ear length and weight, and two SNPs associated with ear row number and length (Khatun et al., 2022). These findings collectively emphasise the significant role of genetics in shaping maize ear characteristics, providing valuable insights for future breeding efforts aimed at enhancing ear weight and overall crop yield.

Dehusked Ear Diameter and Length

The dehusked ear diameter is an important trait to consider as it provides insights into the size and potential yield of the maize ear. A larger diameter indicates a larger ear size, which can accommodate a greater number of kernels and potentially result in higher grain yield. From Table 2, the dehusked ear diameter of maize hybrids varied significantly (p-value ≤ 0.0001), with experimental hybrids JK and GJ exhibiting the largest diameters of 49.76 mm and 50.30 mm, respectively. Experimental hybrids JK and GJ were 15.81% bigger in dehusked ear diameter compared to others, ranging from 42.12 mm to 47.11 mm.

Rahouma and Mahmud (2021) reported a significant difference in dehusked ear diameter, with Pioneer 3084 displaying a diameter of 45.2 mm. Additionally, Carvalho et al. (2021) found that cultivars of P1630 had a dehusked ear diameter of 46.8 mm. In comparison, the current study surpasses these previous findings, reporting even larger diameters of 50.30 mm for experimental hybrid GJ. These results highlight the remarkable potential of the experiment hybrids in achieving larger ear diameters, suggesting their superiority in terms of size and yield potential.

The variation in dehusked ear diameter among hybrids is likely influenced by genetic factors, as different genotypes have distinct genetic backgrounds that contribute to their ear size (Karaşahin, 2022). Furthermore, environmental factors such as climatic conditions during grain filling stages can also affect ear diameter (Zuo et al., 2021). The findings of

this research emphasise the importance of considering dehusked ear diameter as a key trait in maize breeding programs. It plays a significant role in determining the potential yield of maize (Mottin et al., 2021). Therefore, selecting hybrids with larger dehusked ear diameters can contribute to achieving higher grain yields in maize cultivation.

According to Mosharrof et al. (2021), dehusked ear length in maize serves as a critical parameter for determining the overall yield and productivity. Among the experimental hybrids, HJ exhibited a significantly longest dehusked ear length (p-value ≤ 0.001), which was 18.97 cm (Table 2). In contrast, other experimental hybrids (JK (p-value = 0.008), HI (p-value = 0.01), GJ (p-value = 0.02) and GH (p-value = 0.02)) showed significant differences in ear length compared to the HJ hybrid, ranging between 16.26 cm and 17.29 cm. Meanwhile, among the check varieties, GWG888 displayed a significant difference in ear length with 19.63 cm compared to BTL 1 (p-value = 0.02), Pacific 338 (p-value = 0.01), Suwan 5 (p-value = 0.02) and TVDC (p-value = 0.05). In comparison with the experimental and check varieties, both hybrids GWG888 and HJ stood out with the longest dehusked ear lengths compared to the other genotypes, with differences reaching up to 11.97%, highlighting their distinctiveness in this aspect. Their exceptional length sets them apart from the rest of the genotypes in this study (Figure 1).

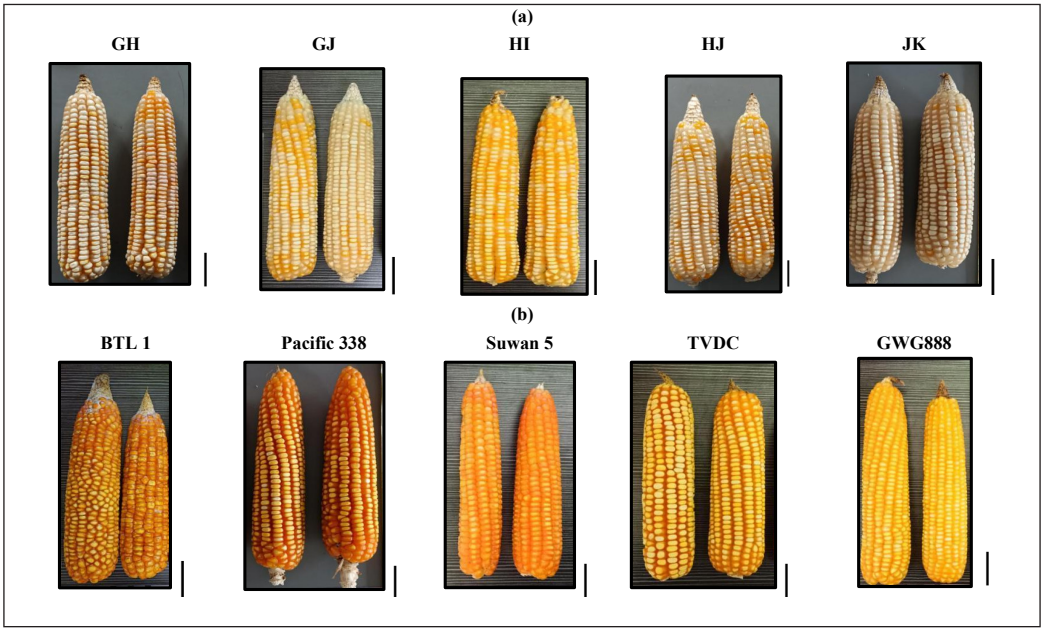

Figure 1. (a) Harvested ears for five experimental hybrids (GH, GJ, HI, HJ and JK); (b) Harvested ears for five check varieties (BTL 1, Pacific 338, Suwan 5, TVDC and GWG888). The line at the bottom right of the picture indicates a rule measuring 3 cm in length

It is important to acknowledge that the ear lengths seen in this study, although remarkable, do not surpass the previously documented record of 21.96 cm in the crossbreeding of M 704 x M 712, as reported in a separate study conducted by El-Hosary et al. (2018). Furthermore, Mousa et al. (2021) found a significant difference of 20.97 cm in length between the cross Gz-8093 x SC131. However, this study aligns with the results obtained by Carvalho et al. (2021), who similarly observed a statistically significant difference in AS 1656 PRO2, measuring 18.10 cm, and the experimental hybrid HJ in this study, measuring 18.97 cm.

The variations in dehusked ear length among the hybrids indicate the distinct genetic composition of these hybrids. Each hybrid carries a unique combination of genetic factors inherited from parental lines contributing to phenotypic variations (Dong et al., 2023). Hence, breeders need to identify the genetic components underlying this trait. However, identifying the genetic components underlying this trait is challenging due to the complexity of the maize genome and the difficulty of cloning ear length quantitative trait loci (Zuo et al., 2021).

Number of Kernel Rows per Ear and Kernels per Row

One of the factors of grain maize production that is of significance includes a high count of kernel rows per ear and kernels per row. Maize is a wind-pollinated plant, and successful pollination and fertilisation of kernels are essential for grain formation. These characteristics significantly impact maize grain yield and can serve as reliable selection indicators for identifying superior maize genotypes (Prakash et al., 2019).

Table 2 reveals that the experimental hybrid JK had the most kernel rows per ear, with 15.6 kernel rows per ear. The difference in the number of kernel rows per ear between JK and other hybrids was 2.2%. Other hybrid results were recorded with a range of 12.4 to 14.8 kernel rows per ear. These results suggest that JK has the potential to produce ears with more kernel rows than other maize hybrids. Furthermore, the number of kernels per row among various genotypes showed significant differences (p-value ≤ 0.0001), as shown in Table 2. Pacific 338, GJ, GWG888 and HJ exhibit significant differences from other hybrids, with the highest value recorded to be 37.3 to 40.2 kernels per row, respectively.

It is interesting to note that the results of this study are consistent with those of other studies. For example, El-Hosary et al. (2018) reported a value of 15.3 for the number of kernel rows per ear in the cross M 712 x M 705; meanwhile, Rahouma and Mahmud (2021) reported a value of 14.3 for the variety Pioneer 3084. In terms of the number of kernels per row, El-Hosary et al. (2018) observed the highest count of 43.0 kernels per row in the cross M 712 x M 705. On the other hand, Rahouma and Mahmud (2021) documented a value of 52.2 kernels per row in the Pioneer 3084 variety.

The recognition that the experimental hybrid JK exhibited the highest number of kernel rows per ear underscores the paramount importance of this trait in enhancing maize productivity. The number of kernel rows per ear directly shapes the formation of grains, underscoring its pivotal role in determining the overall yield. Usually, each ear will form an even number of 12 to 18, where the beginning of the first kernel initiates separation, resulting in the formation of two rows from one (Zhong-zhi et al., 2010). The genetic makeup of a hybrid is the primary determinant of the potential number of rows per ear, with environmental factors playing a comparatively minor role. The establishment of the number of kernel rows per ear occurs rapidly after the initiation of the ear shot, and once determined, no additional rows will develop. This intricate interplay of genetic and environmental factors underscores the swift and decisive nature of kernel row development in maize.

On the other hand, the number of kernels per row trait is also mostly determined by genetic factors, but they can also be modified by environmental conditions. Specifically, a higher number of kernels per row in maize signifies successful pollination and fertilisation processes (Wei et al., 2019). Overall, the results of this study suggest that JK has the potential to produce ears with more kernel rows, while HJ has the potential to produce ears with more kernels per row than other maize hybrids. Further research is needed to determine the factors that contribute to the higher number of kernel rows and kernels per row in JK and HJ.

Grain Weight and Yield

Grain weight and yield are key factors in determining the profitability of maize production. The grain weights exhibited significant variation among the experimental hybrids, as seen by the data presented in Table 3. Although the experimental hybrids had higher grain weight overall, it is worth noting that one of the experimental hybrids, particularly HI, demonstrated the lowest grain weight, measuring at 174.4 g per ear. This observation implies that these genotypes exhibit significant variations (p-value = 0.0003) in grain weight attributes. Surprisingly, there were no statistically significant differences (p-value > 0.1) seen among the experimental hybrid HI, BTL 1 (193.1 g), Suwan 5 (180.4 g), and GWG888 (181.7 g). The observed gap underscores the variability in grain weight and its potential influence on overall crop productivity.

Plants experience elevated levels of photosynthesis when there is an increase in carbon dioxide, resulting in higher production of carbohydrates and biomass (Kellner et al., 2019; Thompson et al., 2017). This will be a directing resource to reproductive structures like ears, resulting in higher ear weights and potential crop yield. However, this process can be interrupted by the environment. Heterogeneous light conditions in maize plants lead to a

reduction in carbon allocation to the ear, resulting in lower yield and decreased proportion of carbon in the ear (Chen et al., 2020). In this study, genetic factors may also contribute to the efficiency of carbon conversion to biomass because the planting area and environment were standardised for each hybrid.

For maize, Table 3 also shows that there were significant differences (p-value = 0.05) in grain yield among the various genotypes. Pacific 338, JK, and GJ had significantly higher grain yields ranging from 10,831 kg/ha to 11,794 kg/ha, compared to other varieties. Among the experimental hybrids, GJ and JK led in grain yield, followed by GH, HJ and HI. Among the check varieties, Pacific 338had significantly higher grain yield than BTL 1 (p-value = 0.03), Suwan 5 (p-value = 0.03), TVDC (p-value = 0.05), and GWG888 (p-value = 0.05).

Maize grain yield is influenced by genetic and environmental factors, including water and nutrient uptake. Proper root development and functioning are crucial for absorbing water and nutrients from the soil. He et al. (2019) found that adequate water and fertiliser can improve photosynthesis and overall maize yield. In addition, the anatomical structure of the ear, comprising its dimensions such as length, diameter, as well as the arrangement of rows and kernels within each row, has a direct influence on the overall grain productivity (Chaurasia et al., 2020; Saritha et al., 2022). Maize cultivars exhibiting larger and more fully developed ears have a propensity to generate grain yields as a result of their capacity to accommodate a higher quantity of kernels (Wei et al., 2019). The role of the ear has been recognised as a crucial determinant of grain yield in several study settings (Jia et al., 2020; Yang et al., 2020). According to the findings shown in Table 3 and Table 2, the check variety TVDC had a relatively high grain weight of 200.1 g per ear. However, it is

Table 3
The effects of genotypes on selected experimental and check varieties on grain weight and yield of grain maize

Genotype	Grain Weight/Ear, g	Grain Yield/Plot, kg/ha
GH	198.8±13.1abc	10009±3040.1bcd
GJ	222.8±20.5a	11794±1101.4a
HI	174.4±19.0d	9276±1013.4def
HJ	216.9±10.0ab	9452±863.4cde
JK	211.9±9.1ab	11158±570.6ab
BTL 1	193.1±13.0bcd	9186±139.7def
Pacific 338	213.3±14.1ab	10831±700.5abc
Suwan 5	180.4±15.7cd	8487±1145.4ef
TVDC	200.1±15.6abc	7859±1343.8f
GWG888	181.7±11.0cd	9240±517.4def
cv	7.435	9.586

Note. Means followed by the same letter are not significantly different between the genotypes by the DMRT test at p-value ≤ 0.05. Values are presented as mean ± standard error (SE). CV refers to the coefficient of variation, which indicates the relative variability of the data

noteworthy that this particular variety also demonstrated the lowest grain yield, measuring 7,859 kg/ha. Besides exhibiting the lowest grain yield, TVDC also showed inferior performance in terms of other ear characteristics, which are often characterised by the lowest values.

Previous studies have shown that the grain yield of AG 9045 RR PRO, a hybrid maize variety, achieved a grain yield of 10,378 kg/ha (Carvalho et al., 2021). Additionally, Pioneer 3084 showcased its potential by yielding up to 7,192 kg/ha (Rahouma & Mahmud, 2021). These outcomes underscore the substantial potential of the experimental hybrids to attain higher grain yields, akin to the benchmark varieties. Consequently, this indicates that these hybrids are well-suited for cultivation and have promising prospects for increasing overall productivity.

Pearson Correlation

Significant relationships among various traits were observed through correlation analysis (Table 4). Firstly, the correlation coefficient at $r = 0.85$ (p-value = 0.02) was found between dehusked ear weight and grain weight, which suggests a strong positive correlation between these two variables. An increase in dehusked ear weight correspondingly results in an increase in grain weight, indicating that larger dehusked ears generally contain more grains. These findings can be supported by other studies that reported a strong correlation between dehusked ear weight and grain weight in maize (Nordin et al., 2018; Prakash et al., 2019).

Secondly, plant height and ear height also indicate a strong positive correlation with the correlation coefficient value of $r = 0.78$ (p-value = 0.05). As the overall height of the plant increases, so does ear height. The study by Singh et al. (2020) further supports this positive correlation, reporting phenotypic correlation coefficients of 0.70 in the maize crop. These findings suggest that taller plants are likely to have higher ear placement, impacting factors such as pollination, ear emergence, harvestability, and optimal sunlight exposure. Plants with greater height possess a heightened capacity to efficiently capture sunlight. Increased sunlight exposure enhances photosynthesis, providing more energy for the development and maturation of the ears and kernels (Yang et al., 2022).

In contrast to the strong positive correlations mentioned above, a weak positive correlation at $r = 0.36$ (p-value = 0.04) was observed between grain yield and the number of kernel rows per ear. While this correlation suggests that an increase in the number of kernel rows per ear will correspondingly result in an increase in grain yield, it is not as strong as the other correlations mentioned. In contrast, Singh et al. (2020) observed a weak phenotypic correlation coefficient ($r = 0.14$) but a strong genotypic correlation coefficient ($r = 0.80$) between the number of kernel rows per ear and grain yield for the maize crop. The differences in findings may be attributed to variations in genetic backgrounds, environmental conditions, or experimental setups.

Table 4
Correlation coefficient estimates among nine traits in maize hybrids

	Plant Height	**Ear Height**	**Dehusked Ear Weight**	**Dehusked Ear Diameter**	**Dehusked Ear Length**	**Kernel Rows Per Ear**	**Kernels Per Row**	**Grain Weight**	**Grain Yield**
Plant Height	1.00								
Ear Height	0.78*	1.00							
Dehusked Ear Weight	0.03	0.02	1.00						
Dehusked Ear Diameter	0.06	0.25	0.68*	1.00					
Dehusked Ear Length	- 0.19	- 0.42*	0.25	- 0.14	1.00				
Kernel Rows Per Ear	0.28	0.26	0.54*	0.65*	- 0.07	1.00			
Kernels Per Row	- 0.11	- 0.04	0.46*	0.23	0.61*	- 0.02	1.00		
Grain Weight	0.13	0.14	0.85*	0.66*	0.09	0.39*	0.44*	1.00	
Grain Yield	0.13	0.29	0.49*	0.61*	- 0.19	0.36*	0.42*	0.59*	1.00

Note. Indicates significant difference by the F test (p-value ≤ 0.05)

Grain yield is considered the main trait of interest for assessing the correlations with other traits. However, there were three traits, namely plant height, ear height, and dehusked ear length, that recorded a non-significant correlation with grain yield. Meanwhile, a moderate correlation was found between grain yield and dehusked ear diameter, with a correlation coefficient of r = 0.61 (p-value = 0.05), which indicates that ear diameter has a significant impact on grain yield, and as the diameter of the ear increases, the grain yield also increases. Similar findings were reported in other studies, where strong correlations were found between ear diameter and yield in maize crop (Greveniotis et al., 2019), as well as other traits (Tandzi & Mutengwa, 2019).

Furthermore, the dehusked ear diameter not only exhibited a positive correlation with grain yield but also demonstrated positive correlations with dehusked ear weight (r = 0.68, p-value = 0.06), number of kernel rows per ear (r = 0.65, p-value = 0.02), and grain weight (r = 0.66, p-value = 0.06). These correlations indicated that as the diameter increases, the dehusked ear weight, number of kernel rows per ear, and grain weight also increase.

CONCLUSION

This study has provided a comprehensive evaluation of the morphological and yield-related traits among a range of experimental and check variety maize hybrids. Significant variability was observed in traits such as plant height, ear height, dehusked ear weight, ear dimensions, kernel arrangement, and ultimately, grain yield, highlighting the influence of genetic diversity among the hybrids. Notably, hybrids such as JK, GJ, and HJ consistently outperformed others across multiple traits, with JK exhibiting the highest number of kernel rows per ear, GJ demonstrating greater dehusked ear diameter and grain yield, and HJ showing the longest ear length and highest grain weight among experimental hybrids. Shorter plant and ear heights observed in hybrids like GH and HJ are aligned with lodging resistance and agronomic preference in high-wind environments, as supported by previous studies. Meanwhile, moderate to high ear heights in high-yielding genotypes suggest that balance, rather than extremes, in morphological traits may be key to optimising yield. The strong positive correlation between dehusked ear weight and grain weight and between ear diameter and grain yield underscores the pivotal role of ear morphology in determining final grain production. Importantly, the genetic basis of many traits evaluated, such as ear length, kernel row number, and grain weight were reaffirmed by correlations and prior findings on heritability and SNP associations. These insights are vital for maize breeding programs aiming to combine high yield potential with desirable agronomic characteristics. While the influence of environmental conditions was controlled in this study, future research should explore genotype-by-environment interactions to validate the performance stability of superior hybrids across diverse agroecological zones.

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DISCLAIMER

There are no artificial intelligence (AI) tools that were used in the preparation of the manuscript.

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Unravelling the Diversity of *Azolla cristata* from the Progo and Opak River Basins based on Inter-Simple Sequence Repeat Marker and Morphological Analysis

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ABSTRACT

Azolla is a floating aquatic plant that offers numerous advantages, including high growth rates and significant protein content, making it a valuable resource for animal feed, fertiliser, and a potential carbon capture agent. Taxonomic analysis and the diversity of *Azolla* in Indonesia remain unclear. Therefore, this study explores the diversity of *Azolla* species in the Progo and Opak river basins, using 20 morphological characters and 10 primers of inter-simple sequence repeat (ISSR). Morphological characters that strongly contribute to differences between genotypes are branching patterns, dorsal lobe shapes, presence of ventral stomata, lobe length, hyaline ratio, and number of branches. The genotypes are identified as *Azolla cristata*, which are synonyms of conspecific species *Azolla mexicana* and *Azolla microphylla*. Among 10 inter-simple sequence repeats (ISSR), UBC 814 and UBC 841 exhibit the high PIC (0.29; 0.28) and RP (19.71; 23.29), respectively. The unweighted pair group method cluster analysis grouped the accessions into two clusters based on their morphological and genetic traits. Diversity indices were moderately high across the two populations, with Nei's gene diversity (He) = 0.230 and Shannon's information index (I) = 0.342. AMOVA revealed that 93% of the overall genetic variation occurred within populations, while only 7% was attributed to differences among populations. The low differentiation suggests high gene flow between the two river basins, likely facilitated by water currents, waterfowl, or humans, effectively maintaining a single large, genetically diverse metapopulation.

Keywords: Azolla cristata, conspecific species, environmental data, morphological characteristic, phenetic relationship, similarity index

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INTRODUCTION

Azolla is an aquatic fern primarily indigenous to warm climates, including tropical and subtropical countries such as Africa, Asia, and the America (Bocchi & Malgioglio, 2010). *Azolla* is referred to as the "green gold mine" due to its numerous advantages (Wagner, 1997). *Azolla* has a very high growth rate of only 2-5 days in multiplication, making it potentially used as a carbon capture agent (Hamdan & Houri, 2022). Moreover, the high protein content in *A. pinnata, A. filiculoides,* and *A. mexicana* makes this aquatic fern a valuable supplement for animal feed, including for chickens, ducks, and fish (Nasir et al., 2022b; Sarah & Ratnam, 2020). Several studies have shown the effectiveness of *A. filiculoides* and *A. pinnata* as a biofertiliser, phytoremediation, and wastewater treatment (Amare et al., 2018; Brouwer et al., 2018; Kadir et al., 2020). The numerous benefits of *Azolla* require adequate conservation efforts to ensure that each type of *Azolla* may be used optimally for its intended function. Hence, the taxonomy of *Azolla* remains controversial to this day, especially within the *Azolla* section, due to species-level similarities and varying characteristics across different geographical and ecological environments (Pereira et al., 2001).

Genus *Azolla* has 7 species that are divided into 2 sections: *Azolla* and *Rhizosperma*. Section *Azolla* consists of *A. microphylla* Kaulf., *A. caroliniana* Willd., *A. mexicana* Schlecht. & Cham. ex K. Presl, *A. rubra* R. Br., and *A. filiculoides* Lam. The section *Rhizosperma* comprises *A. pinnata* R.Br., and *A. nilotica* Decne. ex-Mett (Pereira et al., 2011). However, Evrard & Van Hove (2004), considered *A. mexicana* and *A. microphylla* as a single species based on certain morphological features. Metzgar et al. (2007) and Reid et al. (2006) also classified them as a single evolutionary lineage. The *Azolla* taxonomic controversy over the years is largely due to frequent misinterpretation of morphological characters. Some reproductive characters of *Azolla* spp., such as the morphology and septum number of glochidia around the massulae of microsporocarps, along with the surface characteristics and stratification of the megasporocarp perine (Svenson 1944; Saunders & Fowler, 1992; Perkins et al., 1985), are considered more reliable. However, this reproductive character is difficult to find due to unfavourable environmental conditions (Kumar et al., 2021). Therefore, Pereira et al. (2011) and Kumar et al. (2021) in their research used vegetative characters to distinguish between *Azolla* species.

In Indonesia, the diversity and distribution of *Azolla* is barely studied, with only three species having been identified, namely *A. filiculoides*, *A. pinnata*, and *A. microphylla* (Caruso et al., 2023; Utomo et al., 2019; Wiryati, 2021). This is mainly due to the anthropogenic activities and the wide range of phenotypic variations of *Azolla* that are often misinterpreted by people. *Azolla* displays phenotypic variability in colour, size, and other traits. However, these variances may not directly correlate with alterations in their genotypes but instead indicate environmental factors. For example, heat can induce stress,

typically resulting in pigment shifts between green to reddish-brown due to the synthesis of anthocyanins and a decrease in chlorophyll (Cannavò et al., 2023; Kösesakal, 2014). Moreover, the lack of identification and genetic diversity information arises because studies of *Azolla* in Indonesia primarily focus on the utilisation of *Azolla* in agriculture, such as biofertilization (Jama et al., 2023; Meiriani & Tarigan, 2024), alternative feed for livestock (Susilo et al., 2024), and phytoremediation agents (Lestari et al., 2024). The lack of information on genetic diversity hinders the development of effective conservation strategies.

Therefore, the aim of this study is to describe the diversity of *Azolla* across two populations in the Progo and Opak river basins in Central Java and D.I Yogyakarta using morphological and inter-simple sequence repeat (ISSR). Moreover, we also integrate the ecological data of *Azolla* sampling locations to elaborate on the environmental factors that might affect *Azolla* diversity. ISSR is a simple-to-use marker that has a high level of polymorphism and reproducibility, and is a common molecular marker to analyse genetic diversity in plants (Alaaddin et al., 2022; Dong et al., 2007; Nasir et al., 2022a).

However, research on genetic diversity analysis and the *Azolla* population using ISSR in Indonesia is still very limited. Although genetic diversity analysis using ISSR has been used to analyse other aquatic ferns like *Ceratopteris pteridoides*, the use of ISSR on *Azolla* is limited to verifying homology and distinction between crossbred *Azolla* in China (Dong et al., 2007; Nasir et al., 2022a; Zhong et al., 2011). Other molecular markers used for the analysis of identification and genetic diversity in *Azolla* are RAPD, *trnL-trnF*, ITS1, *rbcL*, *atpB*, *trnG-trnR*, and *rps4-trnS* (Madeira et al., 2016, 2019; Metzgar et al., 2007; Pereira et al., 2011). Specifically, the genetic analysis that has been conducted in Indonesia is identification using *rbcL*, and genetic variation using *trnL-trnF* (Mantang et al., 2018; Najibulloh et al., 2025). The application of ISSR markers in this study, therefore, delivers additional information about the entire genome by focusing on many unidentified nuclear loci. This enables a more detailed examination of genetic variation both within and among populations (Hadipour et al., 2020). Chloroplast and ribosomal markers are often stable and more effective for species identification (Madeira et al., 2019). Conversely, using ISSR enhances the precision of polymorphism assessment in *Azolla cristata*, particularly regarding the connectivity of populations between the Progo and Opak river basins.

MATERIALS AND METHODS

Sampling Area and Plant Material

Field sampling was conducted for 6 months, from June to November 2024. Because *Azolla* is an aquatic plant, purposive sampling was carried out in rice fields and ponds, covering both wild and cultivated species along the Progo and Opak river basins in Central Java and D.I Yogyakarta. The number of sampling locations is proportional to the length of each river system.

Ten samples were collected from the Progo river basin (approximately 138 km), while four samples were collected from the Opak river basin (approximately 65 km). Sampling locations were selected to represent upstream-downstream river gradients and areas where *Azolla* is commonly found, including river-connected agricultural habitats (e.g., rice fields receiving irrigation directly from the river system). The sample size reflects both the spatial distribution and natural availability of *Azolla* along the river basin and ecological scarcity of populations during the dry season (June-November 2024), when reduced river flow leads to fragmented and ephemeral populations. Therefore, this sampling strategy was designed to capture representative genetic variation across river basins rather than to estimate population size or density. This purposive sampling technique was selected for its practicality, offering an efficient and cost-effective method to identify the target population for the study (Lydia et al., 2023; Rebai, 2023). The map of 14 collections of *Azolla* was made with QGIS (Figure 1). Environmental conditions such as light intensity, total dissolved solids (TDS), electrical conductivity (EC), water temperature, humidity (H), and shade condition were recorded as detailed in Table 1.

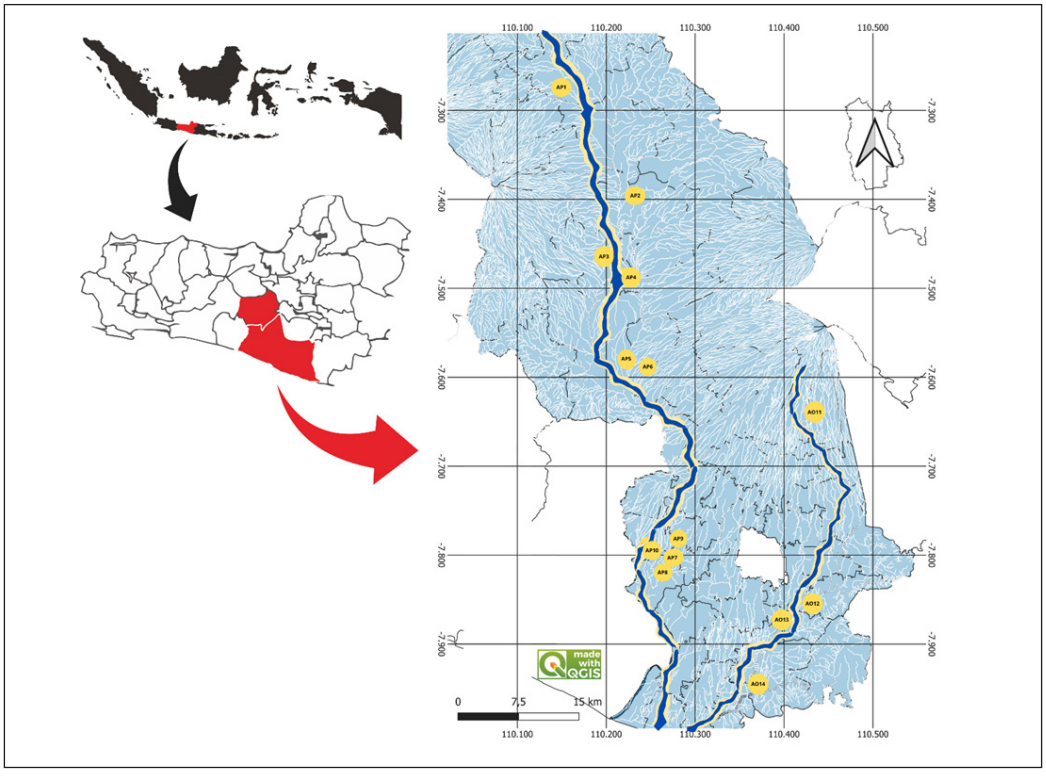


Figure 1. Sampling location of *Azolla cristata* collected from the Progo and Opak river basins, Yogyakarta, Indonesia. Sampling sites AP1-AP10 represent locations along the Progo river basin, while AO11-AO14 represent locations from the Opak river basin

Table 1
Ecological condition of Azolla *collection sites*

Acc.	WT	TDS	EC	LI	H	SC	Latitude, Longitude
AP1	29.10^{ab}	108.0^{b}	216.0^{abc}	49.87^{b}	52.7^{a}	Shaded	7°16'29.40"S, 110° 8'55.50"E
AP2	31.83^{b}	71.7^{a}	143.3^{a}	83.37^{ef}	53^{a}	Unshaded	7°23'48.23"S, 110°13'49.13"E
AP3	37.97^{c}	177.0^{e}	356.7^{de}	96.73^{g}	54^{a}	Unshaded	7°27'50.55"S, 110°12'25.20"E
AP4*	31.83^{b}	79.3^{a}	169.3^{ab}	58.33^{bc}	55.7^{a}	Shaded	7°29'15.02"S, 110°13'13.61"E
AP5	26.57^{a}	127.3^{c}	255.3^{bcd}	57.67^{bc}	56^{a}	Unshaded	7°35'0.09"S, 110°13'35.49"E
AP6	30.20^{ab}	166.0^{e}	321.3^{de}	51.10^{b}	60.3^{ab}	Unshaded	7°35'14.56"S, 110°14'28.89"E
AP7	29.83^{ab}	174.0^{e}	366.3^{e}	59.93^{bc}	61^{abc}	Shaded	7°48'54.57"S, 110°16'4.08"E
AP8	30.63^{ab}	183.0^{f}	368.0^{e}	27.47^{a}	67.3^{bcd}	Shaded	7°48'3.97"S, 110°16'29.18"E
AP9	31.07^{ab}	150.0^{c}	298.7^{cde}	76.80^{de}	70^{cde}	Shaded	7°47'6.36"S, 110°16'44.90"E
AP10	31.27^{ab}	164.3^{e}	348.7^{de}	47.87^{b}	70.3^{cde}	Shaded	7°47'22.10"S, 110°14'48.90"E
AO11	30.10^{ab}	100.0^{b}	197.3^{abc}	52.00^{b}	71de	Unshaded	7°38'20.07"S, 110°26'8.83"E
AO12	29.83^{ab}	182.7^{f}	357.3^{de}	95.00^{fg}	72.3^{de}	Unshaded	7°51'27.30"S, 110°25'57.22"E
AO13*	30.27^{ab}	351.0^{g}	576.0^{f}	69.70^{cd}	75.7^{de}	Shaded	7°52'23.85"S, 110°23'51.84"E
AO14	30.32^{ab}	176.3^{e}	375.0^{e}	66.53^{cd}	79^{e}	Shaded	7°56'45.96"S, 110°22'15.47"E

Note. Acc. accession; AP, Progo river basin; AO, Opak river basin; *cultivated; WT: water temperature (°C); TDS: total dissolved solids (ppm); EC: electrical conductivity (µs/cm), AH: air humidity (% rh); LI: light intensity (Klux); H: Humidity (% rh); SC: Shade condition. Lowercase letters represent statistically distinct subsets at the 0.05 significance level

Morphological Characterisation and Analysis

Morphological identification of *Azolla* species was conducted based on vegetative traits described by Goff (2011), Kumar et al. (2021), Pereira et al. (2011), Svenson (1944), Saunders & Flowers (1992), and along with additional characters supplemented by DMRT-based annotations (Table 2). Observations were performed using an Olympus SZ61 stereomicroscope, a light microscope, and an Optilab® Advance Plus. Quantitative traits were statistically evaluated using one-way ANOVA (Yusuf et al., 2023), followed by Duncan's multiple range test. To assess morphological clustering and trait variation, a dendrogram was constructed using the unweighted pair group technique with arithmetic mean (UPGMA), and principal component analysis (PCA) was executed in Minitab.

Genomic DNA Extraction

Silica gel-dried samples were crushed into a fine powder using liquid nitrogen, and 1 g of polyvinylpyrrolidone (PVP) was added to improve the amount and quality of DNA. The Geneaid DNA isolation kit was used with slight modification. The concentration and purity of the extracted DNA were assessed using a NanoDrop Lite Plus spectrophotometer (ThermoScientific, USA).

Table 2
Morphological parameters in this study

No.	Description	References
1	Sporophyte shape: (0) triangular, (1) starshaped	(Kumar et al., 2021)
2	Imbrication of lobe; (0) slightly imbricate, (1) moderately imbricated, and (2) highly imbricated	(Kumar et al., 2021)
3	Ventral lobe colour: (0) green, (1) pink	(Kumar et al., 2021)
4	Angle of dorsal and ventral lobes: (0) obtuse, (1) acute	(Kumar et al., 2021)
5	Dorsal lobe shape: (0) rounded, (1) sub-round	(Pereira et al., 2011)
6	Rhizome indumentum: (0) glabrous, (1) pubescent	(Pereira et al., 2011)
7	Trichome lobe structure: (0) unicellular, (1) bicellular	(Pereira et al., 2011)
8	Hyalin layers: (0) 3-4 layers, (1) 2-6 layers	(Pereira et al., 2011)
9	Ventral lobe stomata: (0) absent, (1) present	(Pereira et al., 2011)
10	Branching pattern: (0) dichotomous, (1) pinnate	(Svenson, 1944)
12	Trichome lobes arrangement: (0) irregular, (1) in-a-row	(Goff, 2011)
	Hyalin ratio: multistate	(Saunders & Flowers, 1992)
13	Dorsal lobe length: multistate	(Saunders & Flowers, 1992)
14	Dorsal lobe width: multistate	(Saunders & Flowers, 1992)
15	Ventral lobe length: multistate	(Saunders & Flowers, 1992)
16	Ventral lobe width: multistate	(Saunders & Flowers, 1992)

Table 3
Sequence and annealing temperature of ISSR primers used in the study

No.	Primers	Microsatellites Motifs	Annealing Temperature, ºC
1	UBC-814	$(AT)_8YC$	45.5
2	UBC-815	$(GA)_8YT$	50
3	UBC-826	$(AC)_8C$	55
4	UBC-834	$(AG)_8YT$	49.3
5	UBC-841	$(GA)_8YC$	51.2
6	UBC-845	$(CT)_8RG$	50.1
7	UBC-856	$(AC)_8YA$	51.5
8	UBC-860	$(TG)_8RA$	51.5
9	UBC-873	$(GACA)_4$	50.1
10	UBC-880	$(GGAGA)_3$	50.1

PCR-ISSR Amplification

This study utilised ten ISSR primers: UBC-814, UBC-815, UBC-826, UBC-834, UBC-841, UBC-845, UBC-856, UBC-860, UBC-873, and UBC-880 (Table 3). PCR amplification was conducted utilising a Bio-Rad T100 thermal cycler (Bio-Rad, USA) under the following protocols: initial denaturation at 94 °C for 4 minutes, followed by 40 cycles that included 30 seconds of denaturation at 94 °C, annealing at primer-specific temperatures for 60 seconds (Table 3), and extension at 72 °C for 90 seconds. A final extension was performed at 72 °C for 7 minutes. Amplification products were separated by electrophoresis and observed using a GelDoc-UV light transilluminator.

Molecular Data Analysis

Reproducible ISSR bands were recorded as binary data: presence (1) or absence (0) for all samples, resulting in the creation of a binary matrix. The number of scored bands (NA), the number of polymorphic bands (NP), and the polymorphic rate (PR) were recorded. Marker informativeness was assessed by calculating the polymorphic information content (PIC) and resolving power (RP) according to Prevost & Wilkinson (1999). PIC for each primer was determined as shown in Equation (1):

$$PICi=2fi\ (1-fi) \quad [1]$$

Where *fi* is the frequency of band presence at a given locus (Kumar et al., 2014; Roldán-Ruiz et al., 2000). RP was calculated using Equation (2):

$$I_b=1-2\times(0.5-Pi) \quad [2]$$

where Pi represents the proportion of individuals exhibiting a band at a certain locus. Genetic diversity measurements, such as Nei's gene diversity (He), Shannon's information index (I), effective alleles count (Ne), polymorphism percentage (PBB), and molecular variance analysis (AMOVA), were calculated with GenAlEx 6.5 (Freeland et al., 2011; Weising et al., 2005). Phenetic links across samples were illustrated using a UPGMA dendrogram derived from Neighbour Joining distance in MVSP software. Clustering was further validated by Principal Coordinates Analysis (PCoA) utilising GeneAlex.

RESULTS AND DISCUSSION

Descriptive Statistics and Morphological Traits Among the Accessions

The vegetative characteristics used to describe *Azolla* revealed 6 monomorphic traits and 10 polymorphic traits. Although monomorphic characters did not show significant

differences between accessions, they still contributed to the identification of *Azolla* spp. The polymorphic features are eventually utilised for clustering and principal component analysis.

Characteristics of the Sporophyte

All fourteen *Azolla* samples exhibited star-shaped sporophytes with moderate to high levels of leaf imbrication, as illustrated in Figure 2. These morphological characteristics clearly indicate that all accessions belong to the section *Azolla* (Kumar et al., 2021; Madeira et al., 2013). The star-shaped morphology is characterised by overlapping leaf arrangements and closely spaced branches, resulting in angular structures resembling a star, typical of the *Azolla* section (Kumar et al., 2021). Pereira et al. (2011) reported that members of the section *Azolla* exhibit a polygonal frond outline, whereas deltoid shapes are characteristic of the section *Rhizosperma*. The present study provides further clarification by demonstrating that the *Azolla* section also includes forms with pinnately arranged branches, deltoid outlines, and high levels of imbrication (Figure 2). These findings are consistent with earlier observations by Svenson (1944) and Gleason & Cronquist (1991), who reported that *A. microphylla* and *A. mexicana*, both members of the *Azolla* section, develop pinnate structures at full maturity.

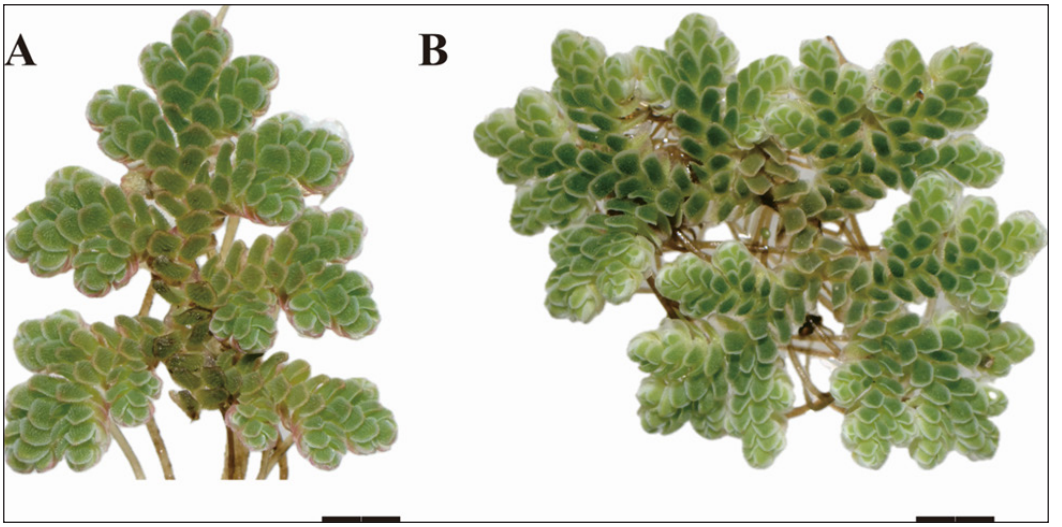


Figure 2. Star-shaped sporophyte with pinnate (A); and isotomous (B) branching. Scale bar = 2mm

Characteristics of Rhizome and Lobe

Rhizome indumentum of glabrous branches (Figure 3A) supported the conclusion that all accessions belong to the *Azolla* section (Pereira et al., 2011). The observed angle between the dorsal and ventral lobes indicates that all samples have an acute angle with the range between 64.5° and 87.6° (Figure 3B and 3C), reinforcing the identification of all accessions into the *Azolla* section (Kumar et al., 2021). This study also revealed that *Azolla* samples are predominantly rounded dorsal lobes, while subround lobes were only identified in the AP4 and AO13 accessions. Pereira et al. (2011) stated that the rounded shapes are possessed by the section *Azolla*, whereas subround are only found in section *Rhizosperma*. However, findings by Kumar et al. (2021) and Svenson (1944) offer a different perspective, noting that *A. mexicana, A. microphylla, A. rubra,* and *A. filiculoides* from the *Azolla* section have slightly acuminated, similar to subround dorsal lobes. Therefore, our findings suggest that dorsal lobe shape is not a reliable trait for distinguishing *Azolla* species. This is likely because the accessions with subrounded dorsal lobes (AP4 and AO13) were collected from cultivated ponds, indicating that environmental conditions may influence this morphological feature. This observation is consistent with the findings of Dunham and Fowler (1987), who concluded that dorsal lobe shape is an unreliable character for species identification due to its variability with developmental stage and environmental factors.

The environment also influences the colour of the ventral lobe. Six of the 14 accessions exhibited a pink ventral lobe, while the remaining samples displayed green colouration. The results showed that unshaded accessions had pink ventral lobes, indicating that sunlight exposure occurred directly on *Azolla* accessions (Table 1). This finding is supported by Sadeghi et al. (2013), who stated that *Azolla* typically maintains a green colour under shaded conditions, while exposure to direct light causes the fronds to turn reddish. In some cases, the pink colour of the ventral lobe can be caused by low nutrients in the *Azolla* media; for instance, AP2, which is in a bright environment with a light intensity of 83.37 klux and low nutrients, has a TDS value of 71.7 ppm and an EC of 143.3 µS/cm, which together indicate low nutrient content in the medium. Total Dissolved Solids (TDS) indicate the concentration of inorganic and organic compounds dissolved in water, while Electrical Conductivity (EC) reflects the ability of water to conduct electricity due to the presence of these dissolved ions. Lower values of both TDS and EC typically correspond to nutrient-poor conditions. *Azolla* tends to be green when in a nutrient-rich environment with sufficient light intensity. A previous study from Cannavò et al. (2023) showed that higher intensity starting from 700 µmol m^{-2} s^{-1} (around 40 kLux) reduces the content of chlorophyll in *Azolla* and makes the lobe of *Azolla* become reddish. Da Silva et al. (2022) also added that *Azolla* could turn red because of the lack of nitrogen, coupled with light intensity. The colour change of the lobes occurred as a result of stress mechanisms caused

by nutrient deficiency or high light intensity, which led to the production of flavonoids such as anthocyanin and phlobaphene (Cannavò et al., 2023; Janes, 1998).

All samples in this study had a bicellular trichome structure (Figure 3E), suggesting they belong to the species *A. caroliniana*, *A. mexicana*, or *A. microphylla* (Pereira et al., 2011). Moreover, all accessions examined in this study displayed a regular (in-a-row) trichome arrangement on the dorsal lobes (Figure 3D), further narrowing the potential identification to *A. mexicana* or *A. microphylla*. This is based on the observation that *A. caroliniana* and *A. filiculoides* typically exhibit irregular trichome distribution on the dorsal lobes (Goff, 2011). This research highlights that the structure of bicellular trichome and the regular or in-a-row arrangement of bicellular trichome in lobus dorsal successfully used to identify *A. mexicana* and *A. microphylla* among species in the *Azolla* section (Chang et al., 2020; Goff, 2011; Pereira et al., 2011).

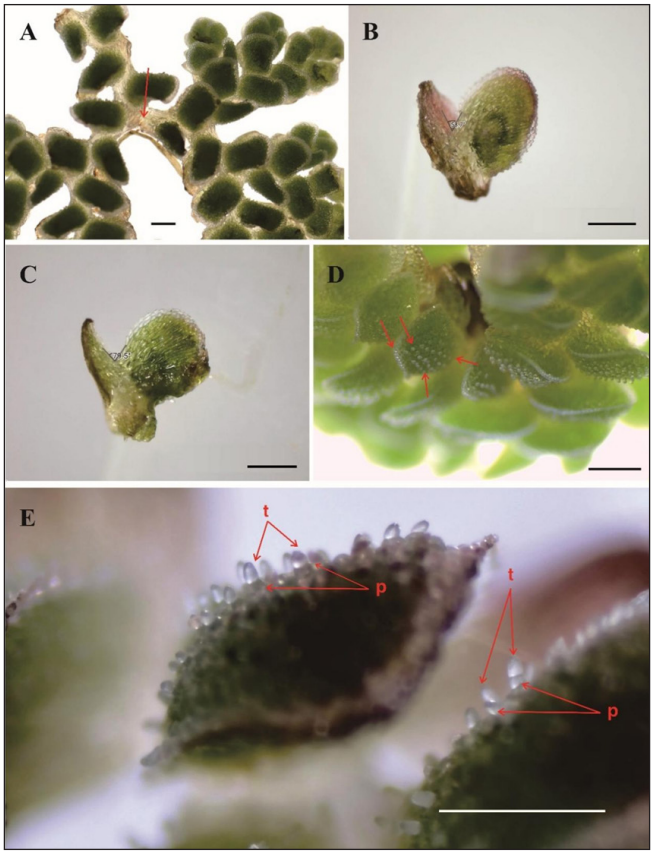


Figure 3. Characteristics of lobes, trichomes and stomata of *Azolla*. Glabrous rhizome (A) acute angle with pink (B) and green (C) ventral lobe. Dorsal lobe with a row arrangement of bicellular trichomes (D) bicellular trichome composed of trichome (t) and pedicle (p) cell (E)

Saunders and Fowler (1992) previously used multivariate statistical analysis on measurements such as the length and width of the dorsal and ventral lobes, as well as the ratio of hyaline layer (Figure 4A), to differentiate *A. nilotica* and *A. pinnata* in the section *Rhizosperma*. This study examined the same measurements using one-way ANOVA for section *Azolla*. The length of the dorsal lobe showed a significant difference ($F = 4.73$; $P < 0.05$), with a range of 0.83-1.32 mm. This result supports the assumption that the sample is likely *A. mexicana* or *A. microphylla* because the lobe length is more than 0.7 mm. Svenson (1944) mentioned that only *A. caroliniana* has a lobe length of about 0.5 mm. The length of the ventral lobe was also noticeably different ($F = 3.96$; $P < 0.05$), measuring between 0.70 and 1.26 mm. In contrast, the width of the dorsal lobe ($F = 0.62$; $P > 0.05$) and the width of the ventral lobe ($F = 1.03$; $P > 0.05$) did not show significant differences. The hyaline layer ratio showed a significant difference ($F = 3.83$; $P < 0.05$) with values ranging from 4.26 mm to 12.1 mm and an average of 8.9 mm.

Accessions AP1, AP4, AP7, AP8, AP9, AP10, AO11, AO12, and AO14 exhibited the presence of stomata on the ventral lobes, whereas other accessions showed few or no stomata in this region (Figure 4B). This characteristic, however, remains inconsistent for defining members of the section *Azolla*. For instance, Pereira et al. (2011) indicated that stomata were exclusively observed in *A. caroliniana*, suggesting limited taxonomic value. However, Chang et al. (2020) and Lumpkin & Plucknett (1980) found contrary results. Chang et al. (2020) mentioned that samples morphologically resembling *A. caroliniana* due to the presence of stomata were actually genetically closer to *A. mexicana.* Moreover, Lumpkin & Plucknett (1980) also reported that *A. filiculoides* has stomata in the ventral lobe. This ambiguity implies that the stomata characteristic alone is not sufficient for species delimitation. Busby & Gunning (1984) supported this idea, stated that the presence of stomata is variable and could appear on both the ventral and dorsal lobes of *Azolla*. The stomata formation depends on specific patterns of unequal cell divisions during the development of *Azolla*.

Considering the ongoing debate surrounding the classification of *Azolla*, this research highlights that all collected samples belong to the conspecific species *Azolla mexicana* or *Azolla microphylla*, synonymous with *Azolla cristata*. This conclusion is supported by the presence of bicellular trichomes, the regular arrangement (in a row) of trichomes on the dorsal lobe, and the length of the dorsal lobe (Ahad et al., 2012; Goff, 2011; Pereira et al., 2011; Saunders & Fowler, 1992). However, this morphological identification should be further validated through molecular characterisation, which is discussed in the following section.

Principal Component and Cluster Analysis Derived from Morphological Traits

Cluster analysis based on morphological characters showed the formation of two main clusters (Figure 5A). Cluster I consisted of two accessions from cultivated samples,

AP4* and AO13*. While the other wild accessions (AP1, AP2, AP3, AP5, AP6, AP7, AP8, AP9, AP10, AO11, AO12, and AO14) were clustered in Cluster II. These two clusters are separated because of morphological differences in the size of quantitative characters, such as the length and width of the ventral and dorsal lobes. Saunders and Fowler (1992) demonstrated the use of these quantitative characters to observe morphological variation. Interestingly, two accessions from the Progo and Opak river basins ("AP" and "AO") merged into one cluster, and only AP4 and AO13 from cultivated ponds separated. Interestingly, two accessions from the Progo and Opak river basins ("AP" and "AO") merged into one cluster, and only AP4 and AO13 from cultivated ponds remained distinct. These results were confirmed with *Principal Component Analysis,* where AP4 and AO13 also separated from others (Figure 5B). In cultivated conditions, the use of organic fertilisers to *Azolla* supplies essential nutrients such as nitrogen (N) and phosphorus (P), which

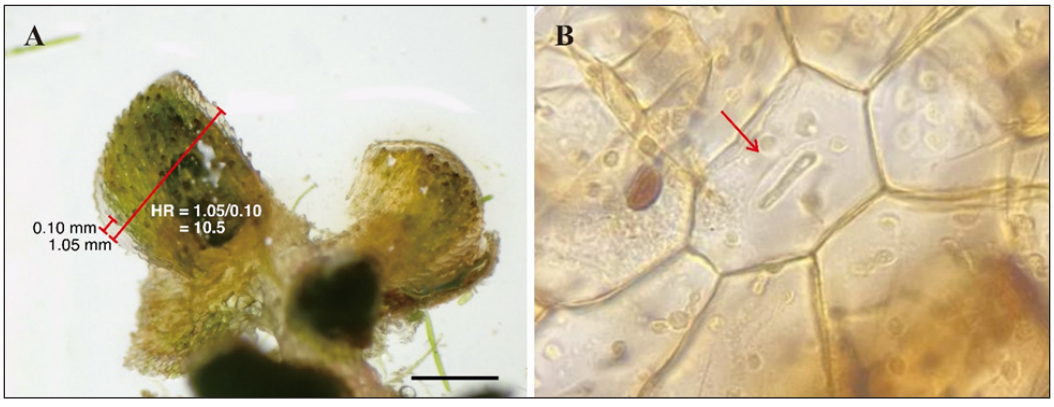


Figure 4. Ratio of hyalin layer (A) and presence of stomata (B)

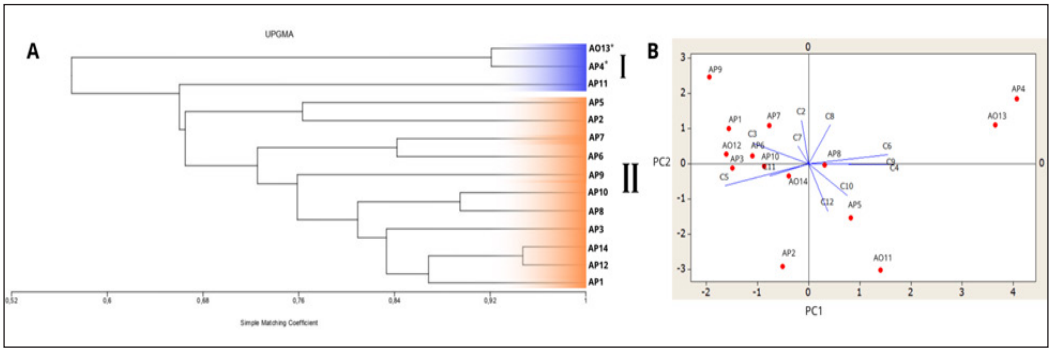


Figure 5. Unweighted pair group method dendrogram among 14 accessions of *Azolla* using morphological markers (A) and principal component analysis of *Azolla* accessions (B)

increase the nutrient content in the plant. This results in enhanced production and growth of *Azolla*, accompanied by an increase in protein content. Moreover, this could affect the geographical distribution, which is different from other wild types of *Azolla* (Azab & Soror, 2020). PCA successfully simplified 18 characters into 11 polymorphic characters, with the highest contributions being C2-branching shape (0.484), C4-branching number (0.459), C5-dorsal lobe shape (0.456), C7-ventral stomatal presence (0.488), C11-hyaline ratio (0.521), and C12-ventral lobe length (0.525) (Figure 5B).

ISSR Analysis

Primer optimisation was conducted by screening 20 ISSR primers synthesised by the University of British Columbia, and 10 of the most optimal primers were obtained. These primers succeed to amplifying 14 *Azolla* accessions, yielding 158 DNA bands and 135 polymorphic bands with sizes ranging from 101 to 1983 bp. The polymorphic band range is 73.3% to 94.12%, with the PIC value averaging 0.23, and the RP value being 18.53 (Table 4). The PCR-ISSR results indicated that the UBC 841 primer generated the highest level of polymorphism at 95.24%, while the UBC 834 primer produced the lowest at 73.3%. Yusuf et al. (2022) claimed that a polymorphic band percentage of 50% is considered high, indicating that the ten primers used in genetic variation analysis are effective. The PIC evaluates the quality and genetic information of primers; PIC values span from 0.0 to 0.5, since they serve as dominant markers. A greater PIC value indicates increased genetic diversity and greater primer effectiveness (Jamil et al., 2022). In addition to PIC, the resolving power (RP) parameter is utilised to evaluate the capacity of primers to distinguish between genotypes (Chesnokov & Artemyeva, 2015; Gilbert et al., 1999; Prevost & Wilkinson, 1999). The primers UBC 814 and UBC 841 are considered the most effective because of their high PR% (95.12; 94.24), RP (19.71; 23.29), and PIC (95.12; 94.24) values, respectively.

The ISSR-based dendrogram analysis classifies the *Azolla* collection into two primary clusters. Cluster I comprises only AO13, while AP1, AP2, AP3, AP4, AP5, AP6, AP7, AP8, AP9, AP10, AO11, AO12, and AO14 categorized inside cluster II (Figure 6). The dendrogram indicates that the two river basins are closely related, as there is no cluster separation based on their location. The only one that separated from the dendrogram was AO13, which came from the cultivated ponds and distinguished itself from cluster II by a similarity value of merely 38%. The highest level of similarity was observed between the accessions of AP6 and AP7 (93%), AP4 and AP5 (90%). According to Jaccard's coefficient, the greatest genetic distance was observed between AO13 and AO11 accessions with a value of 0.685 (Figure 7A). This was followed by genetic distance between AO13 and AO12, AO13 and AP3, AO13 and AP2, with values 0.627, 0.623, and 0.619, respectively.

Table 4
Measured indices of ISSR primers used in Azolla *accessions*

Primer	NA	NP	PR (%)	FS	PIC	RP
UBC 814	17	16	94.12	410-1695	0.29	19.71
UBC 815	11	10	90.90	493-1881	0.21	10.86
UBC 826	13	11	84.61	282-1209	0.25	13.57
UBC 834	15	11	73.30	101-1340	0.19	21.28
UBC 841	21	20	95.24	235-1373	0.28	23.29
UBC-845	15	13	86.67	305-1742	0.25	15.85
UBC-856	17	13	76.47	236-1983	0.20	20.49
UBC-860	15	13	86.67	221-1651	0.22	16.86
UBC-873	17	14	82.35	262-1835	0.28	19.71
UBC-880	17	14	82.35	255-1628	0.17	21
Total	158	135				
Mean		13.5	85.27		0.23	18.53

Note. NA (number of scored bands); NP (number of polymorphic bands); PR (polymorphic rate); FS (fragment size); PIC (polymorphic information content); and RP (resolving power)

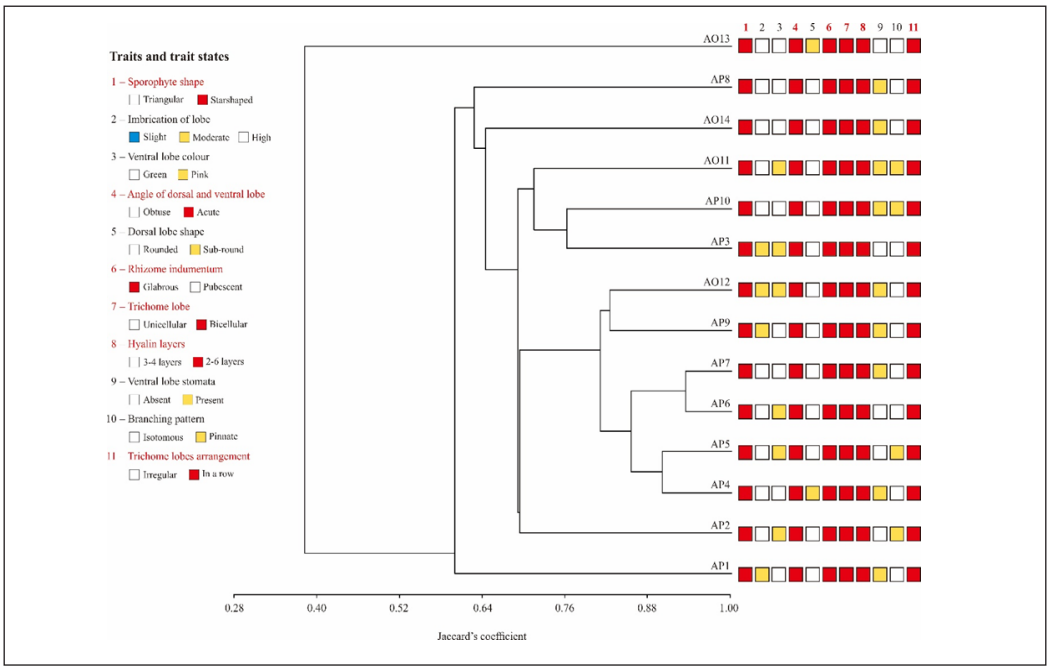
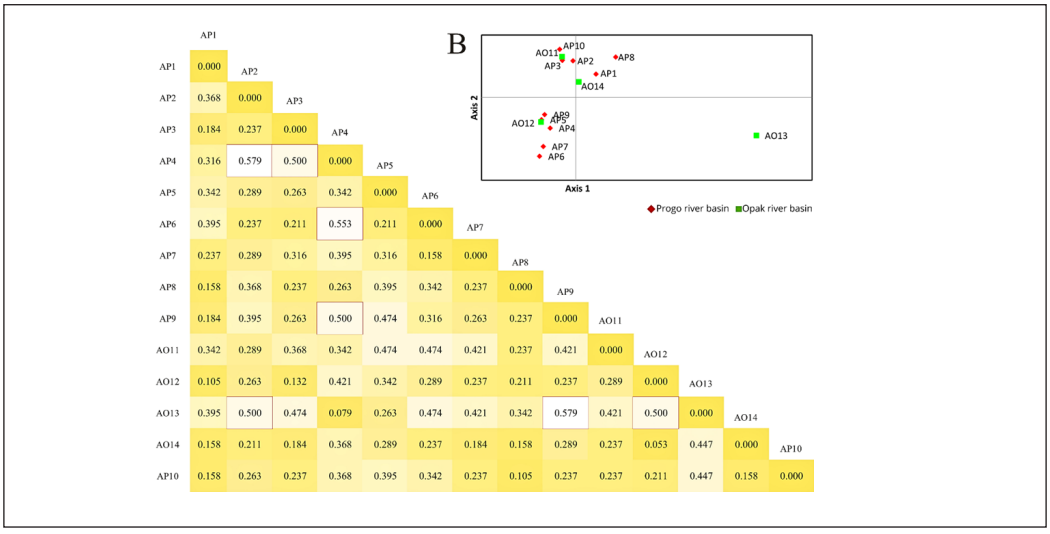


Figure 6. Molecular phenetic tree based on ISSR primers and relatedness to morphological traits. Trait states written in red are the key morphological characters in the populations



	AP1	AP2	AP3	AP4	AP5	AP6	AP7	AP8	AP9	AO11	AO12	AO13	AO14	AP10
AP1	0.000													
AP2	0.368	0.000												
AP3	0.184	0.237	0.000											
AP4	0.316	0.579	0.500	0.000										
AP5	0.342	0.289	0.263	0.342	0.000									
AP6	0.395	0.237	0.211	0.553	0.211	0.000								
AP7	0.237	0.289	0.316	0.395	0.316	0.158	0.000							
AP8	0.158	0.368	0.237	0.263	0.395	0.342	0.237	0.000						
AP9	0.184	0.395	0.263	0.500	0.474	0.316	0.263	0.237	0.000					
AO11	0.342	0.289	0.368	0.342	0.474	0.474	0.421	0.237	0.421	0.000				
AO12	0.105	0.263	0.132	0.421	0.342	0.289	0.237	0.211	0.237	0.289	0.000			
AO13	0.395	0.500	0.474	0.079	0.263	0.474	0.421	0.342	0.579	0.421	0.500	0.000		
AO14	0.158	0.211	0.184	0.368	0.289	0.237	0.184	0.158	0.289	0.237	0.053	0.447	0.000	
AP10	0.158	0.263	0.237	0.368	0.395	0.342	0.237	0.105	0.237	0.237	0.211	0.447	0.158	0.000

Figure 7. Pairwise genetic distance (A) and principal coordinate analysis of 14 Azolla accessions produced by ISSR analysis (B)

Validation by PCoA further supports the genetic distinctions of AO13 compared to other clusters (Figure 7B). The differences of AO13 compared to other samples are supported by the numerous specific bands that appear in the accession across almost all primers, for example, UBC 841 at 1296 bp, 1109 bp, and 336 bp, and UBC 814 at 1132 bp and 817 bp (Figure 8).

AO13, which was morphologically identified as *A. cristata*, consistently clustered separately from other accessions in both morphological and molecular analyses. This distinction may be attributed to phenotypic variation arising from its origin in a cultivated pond environment. AO13 is noticeably different from other accessions because its TDS and EC values are much higher, specifically TDS at 351 mg/L and EC at 576 µS/cm, which are double those of other sample locations (Table 1). Total Dissolved Solids (TDS) indicate the concentration of inorganic and organic compounds dissolved in water, while Electrical Conductivity (EC) reflects the ability of water to conduct electricity due to the presence of these dissolved ions. The higher the TDS and EC values, the more dissolved substances, including nutrients, are present in the water. Despite elevated TDS and EC levels negatively impacting aquatic plants, *Azolla*, an aquatic fern, is often used as a phytoremediation agent, effectively absorbing various heavy metals. The water fern may thrive in nitrogen-deficient circumstances by assimilating atmospheric nitrogen, allowing it to develop without a dedicated nitrogen nutrient supply. As a result, it may thrive in stressful environments, such as polluted water bodies, and accumulate various substances in its vegetative sections (Arora et al., 2006; Umali et al., 2006). Moreover, studies from Kumar et al. (2020) showed

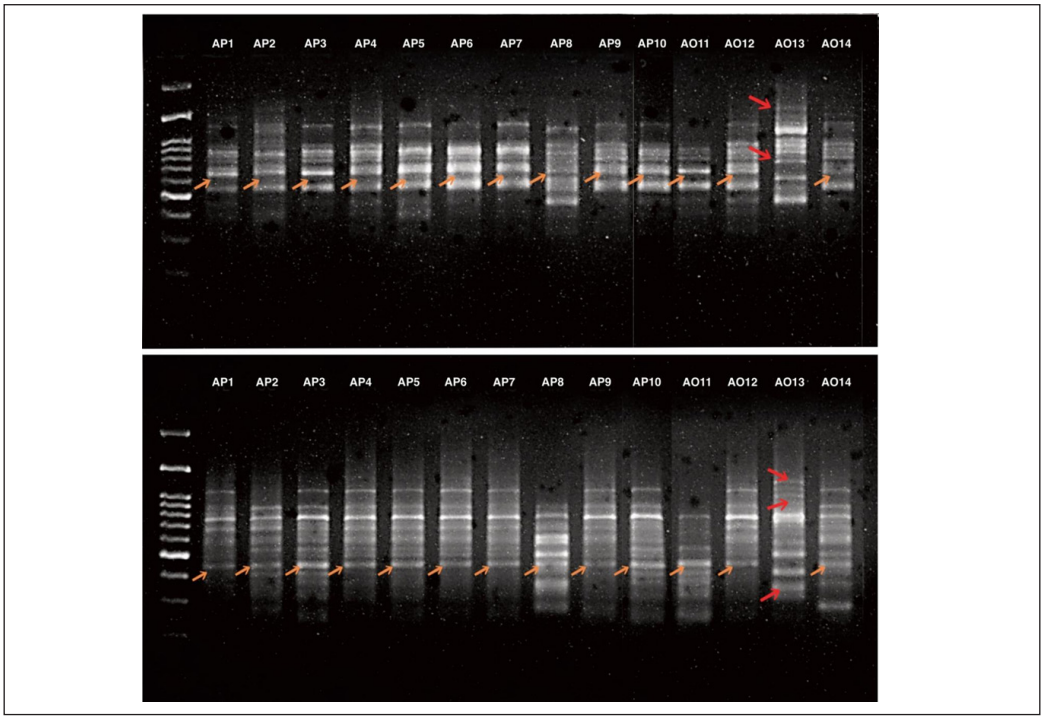


Figure 8. Gel electrophoresis profile of amplified products using primers UBC-814 (top) and UBC-841 (bottom). Red arrows indicate additional bands specific to accession AO13; while orange arrows show unique bands present in other accessions

that *Azolla* can survive in a high TDS environment with a range of 300-900 mg/L and an EC with a range of 500-1300 µS/cm, and even manages to reduce the value of pollutants by decreasing TDS and EC values periodically until day 28. AO13 was separated from other samples, possibly because the prolonged exposure to varying levels of organic and inorganic compounds, such as salts, heavy metals, and other dissolved ions, can lead to intraspecific genetic variation. This is supported by Gupta & Sarin (2009), who found that aquatic macrophytes, where environmental stressors, including nutrient loading, salinity, and heavy metal contamination, could create a change of genetic structure and variation, while analysing it using microsatellite molecular markers.

Through morphology, we agree with Pereira et al. (2011) and Kumar et al. (2021) that some characters could differ between sections *Azolla* and *Rhizosperma* through imbrication, sporophyte shape, rhizome indumentum, angle of lobe, and hyaline layers. In the present study, we confirm that bicellular trichomes, the regular (in-a-row) arrangement of trichomes on the dorsal lobe, and the length of the dorsal lobe could distinguish *A. mexicana* and *A. microphylla* from other species in the *Azolla* section (Goff, 2011; Pereira et al., 2011; Saunders & Fowler, 1992). Initially, we assumed that *A. mexicana* possessed a ventral stomatal lobe while *A. microphylla* lacked one (Chang et al., 2020; Pereira et al., 2011).

However, the ISSR data disclosed an unexpected finding (Figure 6). Accessions that were absent of ventral stomata (AP 2, AP3, AP5, and AP6) joined into one cluster with accessions with the presence of ventral stomata, indicating that the presence of stomata is insufficient for identifying species of *Azolla.* This final result also confirms the findings of Evrard & Van Hove (2004) about detailed microscopic evidence from various cultures and specimens of *Azolla*, showing that *A. microphylla* and *A. mexicana* are morphologically indistinguishable and should be treated as a single species. Previous studies about *Azolla* molecular taxonomy from Madeira et al. (2013), Metzgar et al. (2007), and Reid et al. (2006) supported this theory that *A. microphylla* and *A. mexicana*, based on established nomenclature precedent, are now referred to as *A. cristata* Kaulf.

Environmental Factors

The previous result and discussion suggested that specific environmental factors, like high levels of electrical conductivity (EC) and total dissolved solids (TDS), can affect the quantitative lobe size and intraspecific variation of *Azolla* based on morphological and molecular characterisations (Azab & Soror, 2020; Gupta & Sarin, 2009; Saunders & Fowler, 1992). Moreover, low nutrient content (shown by low TDS and EC) and direct light exposure may have an impact on the colour of *Azolla* leaves (Da Silva et al., 2022; Kumar et al., 2020; Sadeghi et al., 2013).

Moreover, this study discovered that elevation may have an impact on *Azolla's* abundance, even though it did not seem to have an impact on its morphological or molecular characteristics. Only one *Azolla* sample was found at each of the two sampling locations with the highest elevations, AP1 and AO11, which were 648 m and 556 m above sea level (a.s.l.), respectively. These sites were also quite far from the closest *Azolla* population (Figure 1). On the other hand, *Azolla* abundance increased in sites that were geographically close at lower elevations. For example, the samples of AP7, AP8, AP9, and AP10, which are located near one another, were all classified as low-altitude areas with elevations ranging from only 70 to 90 meters a.s.l (Figure 1). The study by Lydia et al. (2023) supports this conclusion by indicating that high altitude adversely affected the dispersal of *Azolla*. Reduced water levels could be the cause of this phenomenon, as *Azolla* thrives on stagnant or relatively slow-moving water. This study examines high-altitude zones (AP1 and AO11) located in mountainous regions, which have rapid downhill water flow. These hydrological conditions possibly limit the growth and survival of *Azolla* in these areas.

Based on the environmental data (Table 1), water temperature ranged from 26.57 °C to 37.97 °C. The suitable water temperature range for *Azolla* survival is approximately 27-35 °C, with the optimal condition around 28 °C (Korsa et al., 2024). The humidity levels at the *Azolla* sampling sites in this study were also supportive of its growth, ranging from 52.7% to 79%. Sadeghi et al. (2013) further supported that the average relative humidity suitable for the growth of *Azolla* spp. ranges around 55% - 83%.

Genetic Diversity

Despite the small sample size, the samples can represent the genetic variation in the two river basins because they were collected to capture the upstream-to-downstream area where *Azolla* was present at the time of sampling. A study by Najibulloh et al. (2025) also used comparable population genetic studies of *Azolla* in the Special Region of Yogyakarta and examined genetic variation and population structure using 11 samples from wild and developed habitats. This previous study successfully identified haplotype patterns and genetic divergence within the region using *trnL-trnF* molecular markers. This finding indicates that, despite limited sampling due to the dry peak season in Indonesia (June-November 2024), molecular markers can effectively detect genetic variation in *Azolla*. Reitsema et al. (2020) and Rivaes et al. (2022) stated that aquatic macrophytes depend totally on their habitat's water supply and are clearly vulnerable to hydrological changes caused by global climate change. Diminished precipitation and reduced water availability in habitats negatively impact the living conditions of macrophytes; conversely, a wetter climate may expand their potential habitats. In river ecosystems, changes in flow patterns have a big impact on macrophytes. Fernández-Zamudio et al. (2010) found that spores of the invasive *A. filiculoides* in a Mediterranean temporary wetland had lower but still significant germination rates after being dried out compared to controls that were always wet. This shows that some spores can survive dry periods and grow new plants when water returns.

The genetic diversity analysis revealed a moderate level of genetic variation within both *Azolla* populations, as indicated by the expected Nei's gene diversity (He = 0.230). The Opak population exhibited higher values of observed alleles (Na = 1.627), effective alleles (Ne = 1.470), Nei's gene diversity (He = 0.266), Shannon's information index (I = 0.392), and percentage of polymorphic bands (PBB = 69.62%), indicating that Opak has higher genetic diversity than the Progo River basin (Table 5). Results from the AMOVA indicated that the majority of genetic variation was distributed within populations (93%) rather than among populations (7%) (Table 6). However, the PhiPT value of 0.067 was not statistically significant ($P > 0.05$), indicating that no statistically significant genetic differentiation was detected between *Azolla* populations from the Progo and Opak river basins. The higher genetic diversity observed in the Opak river basin compared to the Progo river basin is primarily attributed to the genetic difference of sample AO13 compared to other samples from the Opak river basin, which increased the overall variation within the Opak population. This is consistent with clustering analysis that showed the most accessions from both river basins grouped together, except AO13 genetically distinct. However, this differentiation occurred at the individual level and did not result in significant population-level genetic structuring. According to Peakall and Smouse (2012), PhiPT values lower than 0.05 generally indicate negligible or weak genetic differentiation, while higher values reflect stronger population structure. In this study, the PhiPT value of 0.067 with a non-significant

permutation test (P = 0.108 > 0.05) suggests non-significant genetic differentiation between the two river populations. In this study, the PhiPT value of 0.067 with a non-significant permutation test (P = 0.108 > 0.05) suggests that *Azolla* populations from both river systems do not exhibit distinct genetic structure and can be considered part of a single panmictic population. This pattern suggests extensive gene flow or shared ancestry between accessions from the two river basins. This finding is consistent with Al Salameen et al. (2020), who reported that low PhiPT values indicate populations that are not yet genetically structured into distinct groups. Therefore, the ISSR-based results imply high gene flow or shared ancestry among *Azolla* accessions from both rivers, supporting the idea that environmental and geographic factors may influence local genotypic variation (Ahmad et al., 2019).

The high percentage of genetic variation within the population (93%) and the lack of significant population structure are ecologically expected for aquatic plants that possess strong dispersal abilities. *Azolla* is a member of the Salviniaceae family that uses spores as a propagule. According to Reynolds et al. (2015), a propagule is a structure that serves as an agent for reproduction and propagation, including seeds, eggs, spores, statoblasts, and cysts. The propagule found in water ferns, such as *Azolla*, may serve as a potential vector for passive dispersal through various means, including water currents (hydrochory),

Table 5
Genetic variability estimated among two populations of Azolla

Population	Na	Ne	I	He	PBB (%)
Progo River Basin	1.475 ± 0.056	1.335 ± 0.031	0.292 ± 0.023	0.194 ± 0.016	59.49
Opak River Basin	1.627 ± 0.049	1.470 ± 0.032	0.392 ± 0.022	0.266 ± 0.016	69.62
Total	1.551 ± 0.037	1.403 ± 0,022	0.342 ± 0,016	0.230 ± 0,016	64.56

Note. Na (number of alleles); Ne (number of effective alleles); I (Shannon's diversity index); He (Nei's gene diversity); and PBB (polymorphic loci)

Table 6
Analysis of molecular variance (AMOVA) results from 14 Azolla *accessions*

ISSR	df	SS	MS	Est. Var.	Value %	PhiPt	P value
Among populations	1	27.664	27.664	1.413	7	0.067	> 0.05
Within populations	12	235.05	19.588	19.588	93		

Note. df: degree of freedom; SS: sum of squared observations; Est.Var: estimated variance; Value%: percentage of variation; PhiPt: proportion of total genetic variance distributed among populations. P (rand ≥ data): probability based on a permutation test indicating the statistical significance of PhiPT

animals (zoochory), wind (anemochory), and human activities. Such dispersal mechanisms can facilitate the spread of these ferns to new habitats and enhance their ecological success (Casper et al., 2012; Trakhtenbrot et al., 2005), which has potential consequences for gene flow across regions (Ronce, 2007). Previous research has found that *Azolla* sp. fossils are often found in freshwater coastal environments (lagoon or similar) and wetland environments across a wide geographical range. Climate conditions and water flow (hydrochory) facilitate the easy transportation of *Azolla* propagules. Some data showed that *Azolla* fossils found still have microspore massulae attached to the megaspores (De Benedetti et al., 2018). Besides hydrochory, research by Coughlan et al. (2016) showed that *Azolla* was seen to be spread ex-situ with high frequency by mallard ducks (*Anas platyrhynchos*). Bird-mediated dispersal or epizoochory often occurs and significantly contributes to the colonisation, range expansion, and biological invasion. Empirical evidence shows similar mechanisms: waterbirds have been documented dispersing aquatic plant propagules between habitats (Figuerola & Green, 2002; Jones et al., 2020). In contrast, wind had no effect on *Azolla* dispersal (Coughlan et al., 2016).

The Progo and Opak river basins are geographically near and connected hydrologically, offering no significant ecological barrier between them. Engloner et al. (2024) stated that the genetic connectivity between macrophytes increases significantly in canal-connected areas. No significant barrier implies the absence of artificial barriers such as dams that might restrict macrophyte spread and endanger its distribution and persistence (Jones et al., 2020). Nazareno et al. (2021) also supports these statements that the ability of propagules to travel through water likely promotes high genetic connectivity among aquatic plant populations, with limited geographical barriers on their distribution. Besides passive dispersal, *Azolla* is deliberately transferred by humans (anthropogenic), especially for agricultural purposes. *Azolla* is commonly utilised for biofertiliser, compost, livestock feed, and mulch. This usage results in *Azolla* being repeatedly harvested, transported, and washed, which creates numerous opportunities for fragments to reach canals, drainage ditches, or temporary ponds near fields (Korsa et al., 2024; Thepsilvisut et al., 2024).

CONCLUSION

The recent study identified that the *Azolla* accessions in the Progo and Opak river basins are *A. cristata*, which is identical with the conspecific species *A. microphylla* and *A. mexicana,* based on their morphological traits. The use of ISSR DNA fingerprinting markers successfully aided in the patterns of genetic similarity and diversity that support morphological distinctions (Animasaun et al., 2018). The high PIC and RP values seen from the ISSR primers show that they work effectively for *Azolla*. Due to the multidirectional distribution of *Azolla* as a water plant, the genetic diversity remains at a moderate level with low and non-significant genetic differentiation among populations in the Progo and

Opak river basins, suggesting that these populations form one panmictic (randomly mating) population. This pattern is consistent with the aquatic nature of *Azolla* and its potential for gene flow through hydrological connectivity, passive dispersal mechanisms, and human-mediated (anthropogenic) means. This study suggests that further research using additional molecular markers or integrative taxonomic approaches is needed to confirm species boundaries within *Azolla* populations and to clarify population connectivity. This will support sustainable long-term conservation strategies.

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Review Article

Reproduction Technology for Sustainable Portunid Crab's Aquaculture: A Review

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ABSTRACT

Portunid crabs, including the genus *Scylla* and genus *Portunus*, are highly commercial due to their tasty flavour and high meat yield. However, until now, crab demand is still heavily dependent on wild-caught crabs, and the domestication of crabs is necessary for the production of viable seed in hatcheries and the grow-out of portunid crabs. To enhance portunid crabs farming, reproductive technologies should be improved, and this information still needs to be compiled and reviewed. As such, the purpose of this paper is to review reproductive technologies for sustainable portunid crab's aquaculture. This manuscript provides a narrative review of reproduction technology, with an emphasis on the portunid crab from two genera, *Scylla* and *Portunus,* covering literature from 2000 up to 2025, which focuses on practical hatchery applications. Three methods, including hormonal injections, dietary feeding, and manipulation of culture conditions, can be implemented in hatcheries to obtain as many spawned crabs as possible. Pregnenolone, 5-HT, GnRH, methyl farnesoate, prostaglandin, thyroxine and estrogen have been proven to support ovarian maturation in portunid crabs. Formulated diet rich in lipids, adding astaxanthin or using natural diet from fish, blood cockle and squid has been shown to stimulate gonad development and increase spawning rates. Manipulating temperature and photoperiod can also lead to improvements in the egg incubation period and hatching success, with higher water temperatures and longer light periods yielding better results. Through this review, knowledge on reproductive technologies has been gathered and could be further implemented during the farming of portunid crabs, making hatchery-based reproduction more reliable.

Keywords: Feeding, hormone, photoperiod, portunid crabs, temperature

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INTRODUCTION

Crab production is ranked second among crustaceans, after shrimp, and high-value commercial crab consists of the portunid crabs such as mud crab, genus *Scylla* (mud crab) and blue swimming crab, genus *Portunus,* due to their high meat yield and delicate flavour, which satisfy consumer demand for premium seafood (Hidir et al. 2022). Mud crabs are notably known for their large size, reaching 1 kg per crab, especially in male mud crabs due to their muscular chelipeds (Hidir et al. 2021). Mud crabs have been classified via morphometric and allozyme electrophoresis, comprising four species*: Scylla serrata, S. tranquebarica, S. olivacea, and S. paramamosain* (Keenan, 1997). Globally, annual mud crab aquaculture production reached around 408,200 tonnes in 2020, with the vast majority of mud crabs still depending on capture-based fisheries (FAO, 2022). The wholesale price of mud crabs is offered at a high price, reaching 30 USD per kg (Sayeed et al. 2021). Among blue swimming crab species, the most commercially exploited crabs are *Portunus pelagicus* and *P. sanguinolentus*, with an annual global production of around 250,000 tons in 2020 (FAO, 2022). The demand for blue swimming crabs surged in 2022, with an average selling price of 18.6 USD (Setioko et al. 2024).

The early juvenile phase of portunid crabs resides in complex substrates, such as seagrass, as nursery areas to increase survival, while adult portunid crabs are found in coastal habitats, estuarine, and mangrove areas (Vay, 2001; Webley et al., 2009). Nowadays, all these areas are being lost for many reasons, primarily due to the expansion of aquaculture farms, leading to a reduction in wild portunid crabs (Xiong et al., 2024). Additionally, global warming may impact the vulnerable life stages of portunid crabs, especially during the recruitment of larvae and juveniles, which are susceptible to temperature changes, leading to high mortality (Hidir et al., 2021). At the same time, significant warming of ecosystems causes atmospheric CO2 absorption in seawater, reducing pH and impacting the calcification rate of portunid crabs. Until now, crab farming has still relied on wild-caught seed, and uncontrolled fishing of these juvenile crabs has led to overfishing, contributing to a decline in future populations (Fujaya et al., 2021). To overcome these problems, domestication of portunid crabs is necessary for the production of viable seed in hatcheries and the grow-out of portunid crabs. Mature females, which have copulated in the wild, are reared in hatcheries to obtain spawning crabs for seed stock, and reproductive technology must be implemented to achieve this. However, knowledge of reproductive technology is still scarce and needs to be collected. Hence, the purpose of this paper is to review reproductive technologies for sustainable portunid crab's aquaculture. This manuscript provides a narrative review of reproduction technology, with an emphasis on portunid crab from two genera, *Scylla* and *Portunus,* covering literature from 2000 up to 2025, which focuses on practical hatchery applications. Developing reproductive technologies will contribute to stock enhancement and may alleviate the pressing issue of reduced wild stock populations of portunid crabs.

REPRODUCTION TECHNOLOGY OF PORTUNID CRABS

Hormonal Induced

The use of exogenous hormones has shown promise as a replacement for eyestalk ablation and has been highly effective in enhancing growth performance in portunid crabs (Table 1). One of the methods used is the administration of serotonin (5-hydroxytryptamine; 5-HT) to portunid crabs, as there is substantial evidence demonstrating the role of this neurotransmitter in promoting ovarian maturation, especially since this hormone has been found in the ovary of portunid crabs (Saetan et al., 2023). Injecting exogenous serotonin induces the mandibular organ to synthesise methyl farnesoate and the Y-organs to synthesise ecdysteroids, which then act on receptors such as the retinoid X receptor (RXR) and the ecdysteroid receptor (EcR), both of which promote ovarian maturation (Girish et al., 2017). Another hormone that could be used is gonadotropin-releasing hormone (GnRH). GnRH injection can activate the crab adipokinetic hormone/corazonin-related peptide (ACP) receptor expression, which plays a role in lipid metabolism and also upregulates phospholipase A and C, COX, and prostaglandin D, E, and F synthases, all of which are involved in ovarian maturation (Saetan et al., 2023). Additionally, GnRH-injected crabs showed upregulation of other genes, such as LHR and FSHR, whose roles in the activation of reproductive steroid hormones have been confirmed (Saetan et al., 2023). Besides 5-HT and GnRH, two other hormones have been recognized as successfully promoting ovarian maturation in portunid crabs: estrogen and prostaglandins. Estrogen plays a vital role in Vg synthesis in the ovary and hepatopancreas of portunid crabs, and Vg is a yolk precursor for the production of vitellin and egg yolk (Lu et al., 2018). At the same time, estrogen indirectly regulates ovarian maturation by inhibiting the secretion of mandibular organ inhibiting hormone (MOIH) from the brain (Lu et al., 2018). Without MOIH, methyl farnesoate can be synthesised by the mandibular organ and play its role in promoting ovarian maturation. Similarly, the administration of prostaglandins via injection resulted in the elevation of methyl farnesoate and ecdysteroids from the mandibular organ and Y-organ, respectively, and both of these hormones mediate the induction of vitellogenesis in portunid crabs (Swetha et al., 2020).

Iromo et al. (2015, 2021) investigated the use of thyroxine, a hormone that has been rarely studied for its effects on ovarian maturation in portunid crabs. Thyroxine is thought to be involved in gonadal maturation, as its concentration has been shown to increase with advancing ovarian stages in crabs (Iromo et al., 2015). Secreted by the thyroid gland, thyroxine regulates metabolism, particularly protein and lipid metabolism, which may indirectly promote ovarian development through these metabolic pathways (Pucci et al., 2000; Tripathi and Verma, 2003). Another rarely used hormone for stimulating ovarian growth is abalone egg-laying hormone (a neuropeptide secreted by the neural ganglia of molluscs), which is known to trigger egg-laying. Interestingly, Saetan et al. (2017)

demonstrated that this hormone could promote the production of estrogen, thereby inducing vitellogenesis in the hepatopancreas and ovary, resulting in ovarian maturation in female *P. pelagicus*.

Overall, most past studies have used hormones such as pregnenolone, 5-HT, GnRH, methyl farnesoate, prostaglandin, and estrogen to stimulate ovarian maturation, and have primarily assessed hormone-related parameters in the haemolymph. Comparing these hormones to determine which hormone is most effective in inducing ovarian maturation is quite challenging since different hormone-related parameters are being used. In terms of fold change (comparing the optimally hormone-treated group to the control group), prostaglandin appears to produce the highest response among the studies. Although the optimal injection concentration used in Swetha et al. (2020) was relatively high (1.0 µg/g, see Table 1), other studies may have used even higher or similar concentrations but did not achieve optimal results. Since fold change alone may not provide a complete picture, future studies should evaluate additional parameters, such as the total number of spawners and the time taken for crabs to spawn, to better determine the most effective hormone. Until now, only the studies by Farouk et al. (2014), Iromo et al. (2015) and Saetan et al (2023) were more advanced, as they also focused on ovarian maturation stages, although no spawning events occurred.

Feeding

One of the main factors for obtaining ovigerous females at a faster rate is manipulating lipid levels to the optimum in portunid crab feeding, and this can be achieved by introducing formulated pellets for portunid crabs (Table 2). In a study by Aaqillah-Amr et al. (2022a) and (2022b), a formulated semi-moist pellet with a high lipid level (120 g/kg) resulted in higher reproductive performance in terms of HSI, GSI, fatty acids, progesterone and estrogen level in the ovary. As the lipid level increases in the hepatopancreas, it shows that this organ absorbs and stores lipid from the formulated pellet, which then provides enough nutrients to produce vitellogenin for egg yolk formation in the ovary. More specifically, the evidence of nutrient transfer from the hepatopancreas was revealed through histological analysis, where the hepatopancreatic cells were observed to reduce in both tubule diameters and R-cell count, while the ovarian cells demonstrated a higher oocyte diameter. A high lipid diet with adequate nourishment of EPA and DHA levels is not only vital for promoting ovarian maturation in portunid crabs but also for the growth performance of crab larvae (Yin & Kian, 2017). For example, both studies by Aaqillah-Amr et al. (2022b) and Ghazali et al. (2017a) highlighted higher levels of DHA and EPA in the ovaries of crabs fed with scadfish, *Decapterus* sp. and blood cockle, *Anadara granulosa*, which resulted in more advanced ovarian maturation stages. Since crustaceans are unable to synthesise these fatty acids de novo, the requirement for these fatty acids is crucial and must be supplemented via their mother's feeding, where they are then stored for later use by hatching larvae (Aaqillah-Amr et al., 2021).

Table 1
Hormonally induced effect on the reproduction of portunid crabs

Portunid Crab's Species	Hormone	Injection Treatment	Optimal Concentration	Result	Reference
Mud Crab, *S. olivacea*	Pregnenolone	Every 10 days for 60 days	0.01 µg/g	Higher ovary stage and GSI (1.5-fold)	Muhd-Farouk et al., 2014
	5-HT + GnRH	Every 14 days for 28 days	1.5 µg/g (5-HT) 0.05 µg/g (GnRH)	Upregulated of genes related to ovarian maturation, such as FAMeT, ESULT (1.2-fold), progesterone-like protein (2.7-fold) and vitellogenin level (2.4-fold)	Saetan et al., 2023
	Methyl Farnesoate	Every day for 20 days	5.0 µg/crab	Higher GSI and oocyte diameter (2.3-fold)	Muhd-Farouk et al., 2019
Mud Crab, *S. serrata*	Prostaglandin	Every 10 days for 28 days	1.0 µg/g	Higher levels of methyl farnesoate, ecdysteroids, GSI, oocyte diameter (5.4-fold), and vitellogenin (7.3-fold)	Swetha et al., 2020
	5-HT	Every 7 days for 28 days	0.0004 µg/g	Higher levels of methyl farnesoate (1.7-fold) and ecdysteroids (2.3-fold)	Girish et al., 2017
	Thyroxine	Every 5 days for 20 days	0.1 µg/g	Higher GSI (3.9-fold)	Iromo et al., 2021
	Thyroxine	Once	0.1 µg/g	Higher GSI (1.9-fold), shorter time for ovarian maturation (3-fold) and egg incubation period	Iromo et al., 2015
Swimming Crab, *P. trituberculatus*	Estrogen	Every 7 days for 35 days	0.01 µg/g	Higher GSI (1.1-fold) and expression of Vg (2.5-fold)	Lu et al., 2018
Blue swimming crab, *P. pelagicus*	Abalone Egg-laying Hormone	Every 7 days for 28 days	0.5 µg/g	Higher GSI (5.3-fold) and ovary vitellin level (6.6-fold)	Saetan et al., 2017

Note. Serotonin, 5-HT; gonadotropin-releasing hormone GnRH; gonadosomatic index, GSI
In the Results section, 'fold' refers to the comparison between the optimally hormone-treated group and the control group

Table 2
Feeding effect on the reproduction of portunid crabs

Portunid Crab's Species	Diet	Optimal Concentration/ Food Type	Result	Reference
Mud Crab, *S. olivacea*	Formulated pellet: Moist pellet from mangrove clams with different lipid levels	120 g/kg lipid	Higher SGR and GSI in crabs, and higher EPA and DHA in the ovary	Aaqillah-Amr et al., 2021
	Formulated pellet: Moist pellet from mangrove clams with different lipid levels	120 g/kg lipid	Higher GSI, HSI, oocyte diameter, and increased progesterone and estradiol in the ovary	Aaqillah-Amr et al., 2022a
	Natural diet: Scadfish and squid	Scadfish	Higher DHA and EPA in the ovary	Aaqillah-Amr et al., 2022b
	Natural diet: Blood cockle and fish	Blood cockle, *Anadara granulosa*	Higher oocyte diameter	Ghazali et al., 2017b
	Natural diet: Blood cockle and fish	Blood cockle, *Anadara granulosa*	Higher EPA in the ovary	Ghazali et al., 2017a
Mud Crab, *S. serrata*	Natural diet: Blood cockle, polychaete and beef liver	Blood cockle, *Anadara granulosa*	Higher GSI and fecundity	Iromo et al., 2025
	Natural diet: Fish, fish + squid + shrimp, pellet	Fish	Reduced both ovarian maturation and berried period, higher ovary egg diameter, larval hatching	Pattiasina et al., 2012
Mud crab, *S. tranquebarica*	Formulated pellets: Dry pellets from fish meal with different additives from astaxanthin and DHA	Astaxanthin: 50 g/kg DHA: 12.5 g/kg	Higher maturation percentage, spawning rate, GSI, and oocyte diameter	Yin and Kian, 2017
Blue swimming crab, *P. pelagicus*	Natural diet: Squid, *Loligo* sp., blood cockle, *Anadara* sp., fish, *Decapterus* sp. and polychaete, *Neries* sp.	Squid, *Loligo* sp.	Higher larval density	Ikhwanuddin et al., 2015
	Formulated pellet: Different levels of vitamin E	400 IU/kg	Higher egg diameter, fertilisation rate, hatching rate, fecundity	Efrizal et al., 2020

Note. Specific growth rate, SGR; gonadosomatic index, GSI; eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA; hepatosomatic index, HSI; IU, international unit

Besides lipids, another nutrient to consider for nourishing the broodstock of portunid crabs via formulated pellet is astaxanthin, as it acts as an antioxidant, thereby preventing the occurrence of lipid peroxidation during the process of ovarian maturation (Yin & Kian, 2017) (Table 2). Meanwhile, another study by Efrizal et al. (2020) modified formulated pellets by supplementing them with vitamin E at an optimal dose of 400 IU/kg, which resulted in a reduced egg incubation period and improved egg quality.

Besides lipids, protein may play a significant role in stimulating ovarian maturation. Iromo et al. (2025) investigated the effects of different natural diets, such as blood cockle, polychaete, and beef liver, and results showed that crabs fed with blood cockle exhibited the highest GSI and fecundity, perhaps because blood cockle had the highest protein content (46%) compared to beef liver (39%). Although beef liver had a lower protein content, it contained a higher lipid content (26%) compared to blood cockle (7%), which may also support ovarian development; however, excessively high lipid levels could potentially have a slight negative effect on the ovarian maturation of *Scylla serrata*. (Iromo et al., 2025). This finding is supported by Hidir et al. (2018), who reported that wild *S. olivacea* crabs consumed more protein-rich sources such as molluscs and crustaceans during ovarian maturation, and this dietary preference corresponded with higher proteolytic enzyme activity (trypsin) secreted in their hepatopancreas. A study by Ghazali et al. (2017a) found that mud crabs fed with blood cockle exhibited improved ovarian development compared to those fed with fish (*Decapterus* sp.), despite the fish having higher overall protein and lipid content. Similarly, Ikhwanuddin et al. (2015) suggested feeding *P. pelagicus* with squid instead of fish to achieve higher larval density after hatching, even though squid contains less protein than fish. These examples indicate that the type of protein, specifically the amino acid composition in the diet, may play a more critical role in ovarian development than the total protein content alone. Therefore, further research is needed to identify the specific amino acids essential for enhancing ovarian maturation. Additionally, combining this knowledge with an analysis of fatty acid composition may provide stronger evidence of the key nutrients required for successful ovarian development in portunid crabs.

Controlled Environment

Portunid crabs in temperate regions typically grow their ovaries rapidly during the breeding season, which appears to be in summer or spring, and this observation suggests that temperature and photoperiod are the prominent environmental factors regulating portunid crabs' reproduction. For example, the mud crab, *S. paramamosain*, in temperate regions develops its gonad and becomes ready to spawn during the warmer months of spring (Zeng et al., 2007). This also occurs in tropical regions, as proven by a study by Babita (2019), which found that gonadal indices increased with temperature (26.5-37 °C) and prolonged photoperiod (12.2-13.9 light hours) from April to August. Hence, from these observations, it can be concluded that higher temperatures with longer photoperiods may induce ovarian

growth. The term “higher temperature” refers to temperatures considered high relative to the species' natural habitat, and vice versa. For example, the optimal temperature in tropical regions ranges from 30-31 °C, while in temperate regions it is lower, around 21 °C, though this is still considered a high temperature relative to the natural conditions of temperate species (Table 3). Table 3 also summarises several previous studies that have manipulated temperature in captivity to examine its effects on the reproduction of portunid crabs.

Hamasaki (2002) showed that the spawning percentage reached 100% under higher temperatures (18 °C - 26 °C). Moreover, higher temperatures shortened the incubation period for egg development in portunid crabs, as Hamasaki (2002) found a reduction of 14 days in the incubation period when the crabs were exposed to 25 °C compared to a 20 °C treatment. Higher temperatures also aided in fertilisation and hatching, as shown in a study by Efrizal et al. (2006), which found that blue swimming crabs incubated at 28 °C - 30 °C demonstrated the highest fertilisation and hatching rates. Premature egg release by crabs is a common issue in hatchery conditions; therefore, maintaining an optimal temperature is essential to ensure the hatching success of broodstock. Hidir et al. (2021) found that at a higher temperature (30 °C), the hatching success rate reached approximately 75%. While at lower (26 °C) or excessively high temperatures (32 °C), hatching often failed, with success rates dropping to only 36% - 37%. The same study reported that higher temperatures at 31°C are necessary to create optimal conditions for larval survival (more than 66%) and growth. However, if an inappropriate temperature is applied to portunid crabs, either too low or too high, abnormal cell division is observed, and egg development is retarded at the gastrula stage (Zeng, 2007), along with a reduction in egg membrane formation (Efrizal et al., 2006).

Incubating portunid crabs under a longer photoperiod (more than 14 hours) has been shown to promote advanced gonadal development. The influence of longer photoperiods on ovarian growth was successfully demonstrated by Babita et al. (2019), Hamasaki et al. (2004), and Kim et al. (2010), where extended photoperiods (13 h, 14 h, and 15 h, respectively) stimulated gonadal development and resulted in higher percentages of ovigerous females (Table 3). The mechanism behind the effect of light remains unclear and therefore requires further investigation, but only one study reported the influence of pigment-dispersing hormone (PDH) in gonad development. Photoperiodic daily rhythmicity was observed to influence the X-organ in the crab's eyestalk to synthesise this PDH hormone (chromatophore-activating neuropeptide) (Huang et al., 2014). This PDH is also related to ovarian maturation in portunid crabs, as the PDH expression level increases in the eyestalk and ovaries during different stages of ovarian development (Huang et al., 2014). Both observations suggest a possible role of this hormone in regulating ovarian growth. Overall, the information regarding temperature and photoperiod from natural wild habitats and previous captivity experiments is pivotal in aquaculture to enhance culture protocols for accelerating portunid crab spawning.

Table 3
Temperature and photoperiod effects on the reproduction of portunid crabs

Portunid Crab's Species	Climate	Optimal Temp (°C)	Optimal Photoperiod (h)	Result	Reference
Mud Crab, *S. paramamosain*	Temperate	N/A	-	PDH expression aligns with both ovarian maturation and photoperiod, revealing a link between photoperiod and ovarian maturation	Huang et al., 2014
	Temperate	18-26	-	Reduced incubation period of eggs	Hamasaki, 2002
	Tropical	30	-	Reduced embryonic stage duration	Zeng et al., 2007
Mud Crab, *S. serrata*	Temperate	29		Reduced egg incubation period, higher zoea, megalopa and crablet survival, reduced larval incubation period	Hamasaki, 2003
Mud Crab, *S. tranquebarica*	Tropical	30-31	-	Higher hatching success, egg diameter, higher survival and growth for zoea and megalopa, higher crablet production	Hidir et al., 2021
Blue Swimming Crab, *P. pelagicus*	Tropical	30	-	Higher fertilisation rate, hatching rate and reduced egg incubation period	Efrizal et al., 2006
Marine Crab, *P. sanguinolentus*	Tropical	22-28	13	Higher gonad index	Babita, 2019
Swimming crab, *P. trituberculatus*	Temperate	21	14	Earlier spawning	Hamasaki et al., 2004
	Temperate	27-29	-	Higher hatching rate, reduced T-AOC, SOD, GSH, CAT, and MDA	He et al., 2022
	Temperate	20 °C	15	Higher GSI, higher ovigerous females	Kim et al., 2010

Note. Total antioxidant capacity, T-AOC; superoxide dismutase, SOD; glutathione GSH; catalase CAT; and malondialdehyde, MDA

CONCLUSION

The demand for portunid crabs has significantly increased, and hence, the domestication of portunid crabs is vital for the production of viable seed in hatcheries and the grow-out of portunid crabs. To support this domestication effort, a better understanding of reproductive technologies is essential; hence, through this review, knowledge on reproductive technologies has been gathered and could be further implemented during the farming of portunid crabs. Reproductive crab performance could be improved through three methods: hormonal injection, feeding, and manipulation of temperature and photoperiod. These methods accelerate ovarian maturation in portunid broodstock, making hatchery-based reproduction more reliable. This enables a continuous supply for farming and consumption while reducing pressure on wild populations.

In the future, several improvements can be made. For hormonal studies, additional parameters such as the total number of spawners and the time taken for crabs to spawn should be included to better determine the most effective hormone, although these parameters may extend the duration of the experiment. For feeding studies, further research is needed to identify specific amino acids that are essential for enhancing ovarian maturation. Regarding culture conditions, there is a lack of studies on the effect of photoperiod on ovarian maturation in crabs. This area should be further explored, and the use of modern technologies could allow not only for manipulation of photoperiods but also the adjustment of light spectra, using different light colours to potentially enhance reproductive development. The mechanism behind the effect of light remains unclear and therefore requires further investigation.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

AUTHOR CONTRIBUTIONS

Ariffin Hidir designed the study, gathered information, analysed data and drafted the paper. Aaqillah-Amr reviewed the paper. Mhd Ikhwanuddin drafted, reviewed the paper and was involved in funding acquisition, project administration, and supervision.

ETHICAL APPROVAL

Ethical approval is not required as no experiments were conducted in the production of this research paper.

DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are included within the paper. No additional data are available.

LIST OF ABBREVIATIONS

5-HT	:	5-hydroxytryptamine
ACP	:	adipokinetic hormone/corazonin-related peptide
DHA	:	docosahexaenoic acid
EcR	:	ecdysteroid receptor
EPA	:	Eicosapentaenoic acid
GnRH	:	gonadotropin-releasing hormone
GSI	:	gonadosomatic index
HIS	:	hepatosomatic index
MOIH	:	mandibular organ-inhibiting hormone
RAS	:	recirculation aquaculture system
RXR	:	retinoid X receptor
SGR	:	specific growth rate

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Selangor Public Perceptions of Neutering and Preventive Health Measures for the Health of Cats and Dogs

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ABSTRACT

The well-being of companion animals is heavily dependent on public understanding and adoption of preventive health measures, such as neutering, vaccination, and antiparasitic treatments. This study investigated the knowledge and perceptions of Selangor residents regarding these practices and identified the demographic factors influencing their views. A cross-sectional survey was conducted using online and paper-based questionnaires, which covered demographic details, pet management practices, knowledge and perception on veterinary care, and educational intervention. Data were analysed using the IBM SPSS Statistics, employing descriptive statistics and inferential analysis (e.g., Linear Regression and Spearman's correlation). A total of 381 Malaysians residing in Selangor participated in this survey. Findings showed that most respondents were female (n=281, 74.5%) and aged between 21-30 years old (n=172, 45.1%). Most respondents were also pet owners (n = 351, 92.1%), with the majority having cats (n = 306, 87.2%). Results showed that 97.4% agreed that pets should be vaccinated, and 96.9% of respondents were aware of the importance of neutering, despite 79.5% (n = 303) being concerned about pain after neutering. Furthermore, although 74% (n = 282) of respondents were aware of recommended antiparasitic regimens, adhering to these practices was inconsistent. Age (p = 0.045) and education level (p = 0.006) revealed significant influence towards knowledge on preventive healthcare, meanwhile gender (p = 0.010) showed significance regarding the

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perception on neutering and preventive healthcare. Selangor residents showed a moderate level of knowledge regarding pet preventive healthcare. Given that most respondents rely on veterinarians as their primary information source, future educational campaigns led by veterinary professionals are essential to improving animal health and welfare in Malaysia.

Keywords: Animal welfare, antiparasitic treatments, knowledge, Malaysia, neutering, preventative healthcare, vaccination

INTRODUCTION

Cats and dogs have been increasingly popular as pets, specifically in Malaysia, making them the top two most abundant terrestrial animals among Malaysian pet owners, with over 402,500 and 795,000 dogs and cats, respectively, being cared for in 2018 (Munir et al., 2023). Owning pets entails taking responsibility for their physical and mental welfare, including ensuring a high quality of life through therapeutic and preventive healthcare (Ho et al., 2021; Meier & Maurer, 2022). However, there are also negative aspects associated with rising levels of pet ownership, which include the rise of pet overpopulation, transmission of infectious diseases (e.g., zoonotic diseases and venereal diseases), and pet neglect. To mitigate these issues, veterinary associations such as the American Veterinary Medical Association and American Animal Hospital Association (AVMA & AAHA, 2020) have come up with a recommended neutering and preventive healthcare guideline, which will aid in providing the best care for animals.

According to AAHA and AVMA (2020), preventive healthcare is a multi-faceted approach to screen cats and dogs and provide them with vaccination, deworming, and ectoparasite control to prevent and manage disease outbreaks (AVMA & AAHA, 2020). Responsible pet ownership emphasizes on prevention of diseases for cats and dogs for their welfare and well-being. According to the guidelines proposed by AAHA and AVMA (2020), it is stated that cats and dogs should receive at least an annual checkup, with vaccinations, deworming and flea control according to the recommended schedule. Vaccination is crucial in developing cats and dogs' immune systems and providing herd immunity towards transmissible infectious diseases (Dodds, 2021); therefore, it is of importance in improving pets' overall quality of life. However, a significant gap exists in the Malaysian literature regarding this topic, as comprehensive studies on public adherence to these guidelines have yet to be carried out.

In countries such as the United Kingdom, preventive healthcare is one-third of the reasons for visits to veterinary practices, and this shows the perception of UK pet owners regarding the health of pets (Evason, 2021). Meanwhile, in New Zealand, the majority of pet owners believed that all cats and dogs deserve important pet care practices such as adequate housing, regular worming and ectoparasite treatments, microchipping, and vaccinations (Forrest, 2023).

In contrast, this level of awareness regarding animal welfare and health has yet to be realised in Malaysia. The control of parasites (e.g., fleas, mites, and ticks) and endoparasites (e.g., heartworm) is important as a preventive method from parasite-induced pathologies. In fact, according to a report by the Veterinary Research Institute, Malaysia (Masrin et al., 2019) indicated that up to 16% of pet dogs and 76% of stray dogs harbour ectoparasites and endoparasites such as *Ancylostoma sp*., *Giardia sp*., and *Demodex sp*., which can spread rapidly through horizontal transmission. Furthermore, certain parasites such as *Toxoplasma sp.,* are also known to be zoonotic and can be transmitted to pregnant females, which can lead to foetal loss (Masrin et al., 2019).

Despite significant research available on preventive healthcare and neutering among cats and dogs (McKenzie, 2010; Oliveira-Martins et al., 2023), there remains insufficient data and knowledge in the Malaysian context, specifically involving the public's perception. Studies from countries like New Zealand and the United Kingdom show a positive attitude and perception, which is due to support from having high levels of public education regarding animal welfare and health (Downes et al., 2014; Forrest et al., 2023). Neighbouring Southeast Asia countries, such as Singapore, also highlight positive pet ownership values, with the help of effective public education targeting pet owners (Gunaseelan et al., 2013). These measures (e.g., neutering, vaccination, and antiparasitic control) have been extensively implemented among small animal practitioners worldwide (Mueller et al., 2018). However, there are still a few barriers in providing sufficient pet care (e.g., lack of commitment, resources) that need to be explored. These include the ethical views, socioeconomic status, and the understanding of animal welfare (Munir et al., 2023; Zaini et al., 2023).

In comparison to other regions, limited research has been conducted to properly understand the values and perspectives that Malaysians, especially citizens of Selangor, hold regarding animal healthcare. A study conducted in Putrajaya, Malaysia, revealed that there are poor vaccination rates and infrequent veterinary visits among pet owners (Debbra et al., 2019). However, there is not yet any research that studies the factors and barriers influencing these trends. According to a study conducted by Tan et al. (2021), Southeast Asian countries have less public awareness regarding animal welfare and also limited access to veterinary care in comparison to Western countries such as the United Kingdom (Tan et al., 2021).

Consequently, in an urban state such as Selangor characterised by a diverse population and socioeconomic values, this research provides insight into the current situation regarding Malaysians' perspectives on animal healthcare. This study aims to determine the knowledge, perception and practice of the Selangor public regarding neutering and preventive health measures, as well as the demographic factors influencing their perception, for the health and well-being of cats and dogs.

MATERIALS AND METHODS

Ethical Approval and Consent

Ethical approval was granted by the Ethics Committee for Research Involving Human Subjects of Universiti Putra Malaysia (JKEUPM) on 9th May 2024 (Reference number: JKEUPM-2023-805).

Questionnaire Development

A cross-sectional, questionnaire-based study was conducted during a period of 5 weeks from 28th July 2024 until 1st September 2024 to assess the perception and knowledge of the Malaysian public regarding preventive healthcare for cats and dogs in Selangor, Malaysia. The questionnaire was developed and adapted from previous studies and modified according to the suitability and purpose of this study. The questionnaire consists of five sections (Table 1), (i) demographic details (e.g., gender, age, education level, profession, household income, and pet ownership details) (ii) knowledge on preventive healthcare and neuter, (iii) pet management practice (e.g. frequency of bringing pets to the veterinary clinic for preventive healthcare), (iv) perception on preventive healthcare and neutering, and (v) educational intervention in order to gauge the most preferable medium of approach to educate the Malaysian public.

The questionnaire underwent a thorough pilot testing phase, involving a total of 20 respondents and several academicians and researchers, aimed at meticulously scrutinising aspects such as grammar, time requirements, and other pertinent factors. For instance, the definition of neutering was prepared to improve the understanding of the word's use in the study.

Table 1
Summary of questionnaire

Section	Question (Summary)	Type of Question
Demographic details	Gender, age, education level, profession, household income, and pet ownership details	Multiple choice (single answer) question
Knowledge on preventive healthcare and neuter	Questions relating to vaccination, deworming, flea control and neuter (i.e., schedule, roles for each stated preventive healthcare)	Multiple choice (single answer) question
Pet management practice	Pet management style, frequency of bringing pets to the veterinary clinic for preventive healthcare	Multiple choice (single answer) question
Perception on animal management	Perception on preventive healthcare and neutering	Participants rated 9 questions on a 1-5 Likert scale (Strongly Disagree to Strongly Agree)
Educational intervention	The most preferable medium of approach to educate the Malaysian public	Multiple choice (multiple answer) question

Reliability analyses were conducted, and the Cronbach's alpha value was $\alpha = .87$ indicated high reliability; thus, no items were removed prior to the main study.

Participants Recruitment

A total of 381 respondents voluntarily participated in this study by convenience sampling. Participants' criteria include pet owners and non-pet owners, current residents of Selangor, Malaysia, and able to understand Bahasa Malaysia and English. The questionnaire was available physically (paper-based) and digitally on Google Form and distributed through online social media platforms (e.g., WhatsApp, Telegram, Facebook, Instagram).

Statistical Analysis

Data collected from the questionnaires were tabulated into Microsoft Excel and were analysed using IBM SPSS Version 29. The Kolmogorov-Smirnov Test was done to check the normal distribution of the dataset, which shows that it is not normally distributed. Descriptive analysis was done to obtain the frequency, median, and standard deviation of the data, and inferential analysis, such as Linear Regression and Spearman's correlation, was utilised to interpret the relationship between the demographic variables, knowledge, and perception of the public regarding this study. Multiple Linear Regression analysis was conducted to evaluate the impact of demographic factors on knowledge scores. Despite the non-normal distribution of the raw scores, the regression model was deemed appropriate given the sample size (N = 381), which satisfies the assumptions of the Central Limit Theorem for the normality of residuals (Pek et al., 2018).

RESULTS

Demographic Details

A total of 381 respondents from Selangor, Malaysia, participated in this questionnaire. Results showed that most respondents are female (74.5%, n = 281) and aged between 21 to 30 years old (45.1%, n = 172). The majority of the respondents had a bachelor's degree (57.5%, n = 219), worked as a private company staff (38.6%, n = 147), resides in a terrace house (54.1%, n = 206), and comes from a B40 household (59.6%, n = 227) (Table 2).

Pet Management Status

Table 3 represents data on pet ownership status, where out of 381 total respondents, up to 92.1% were pet owners. Most respondents owned cats (87.2%, n = 306), followed by dog owners (9.1%, n = 32), and lastly both cat and dog owners at 3.7% (n = 13) (Table 3). Out of the 351 pet owners, more than half had more than 5 years of experience in pet care, with most preferring to keep their pets fully indoors, and the majority stated that their pets had been neutered.

Table 2
Descriptive statistics of demographic details of respondents

	Frequency (n)	Percentage (%)
Age		
Below 18	3	0.8
19 - 20	40	10.5
21 - 30	172	45.1
31 - 40	92	24.2
41 - 50	46	12.1
>50 years old	28	7.3
Gender		
Female	281	74.5
Male	96	25.5
Education Level		
No formal education	2	0.5
Secondary school	64	16.8
Diploma	74	19.4
Bachelor's degree	219	57.5
Postgraduate	22	5.8
Profession		
Student	97	25.5
Government staff	43	11.3
Private company staff	147	38.6
Self-employed	56	14.7
Retired	5	1.3
Unemployed	33	8.7
Household Income		
B40 (below RM4850/ month)	227	59.6
M40 (RM4851-10970/ month)	128	33.6
T20 (more RM 10971/ month)	26	6.8
Type of Residence		
Apartment	121	31.8
Terrace	206	54.1
Bungalow	28	7.3
Other	26	6.8

Table 3
Pet ownership status and pet management details

	Frequency (n)	Percentage (%)
Own Pets		
Yes	351	92.1
No	30	7.9
Type of Pet		
Cat	306	87.2
Dog	32	9.1
Both	13	13.0

Table 3 (*continued*)

	Frequency (n)	Percentage (%)
Pet Management Style		
Fully indoor pet(s)	175	49.9
Outdoor pet(s)	33	9.4
Semi-roamer pet(s)	143	40.7
Years of Experience		
No experience	4	1.1
Less than a year	11	3.1
1-2 years	48	13.7
3-4 years	70	19.9
>5 years	218	62.1
Pet Neuter Status		
My pet is neutered	188	53.6
My pet is not neutered	30	8.5
I intend to neuter my pet(s)	53	15.1
Some of my pet(s) is neutered while some are not	80	22.8

Knowledge on Preventive Healthcare and Neutering

More than 96% of respondents displayed knowledge and awareness of the importance of neutering and vaccination for their pets' health (Table 4). A significant number of pet owners also stated that they are aware of the recommended preventive healthcare programme, such as vaccination, deworming, and flea control schedules. As for neutering, the majority agreed that the main purpose is to control and decrease the overpopulation of stray, however as for their concerns regarding neutering and spaying, 35.4% of respondents were concerned about pain during the procedure, followed by 26.2% were worried regarding postoperative wound complications after the ovariohysterectomy or orchiectomy surgery.

Table 4
General knowledge and awareness on preventive healthcare and neuter

	Frequency (n)	Percentage (%)
Awareness on Neutering and Spaying		
I know what neutering and spaying is	369	96.9
I don't know what neutering and spaying is	11	2.9
Awareness on Vaccination		
Pets should be vaccinated	371	97.4
Pets do not need to be vaccinated	10	2.6
Knowledge of Vaccination Schedule		
I am aware about vaccination schedule	299	78.5
I am not aware about vaccination schedule	79	20.7
Knowledge of Deworming and Flea Control Schedule		
I am aware of the deworming and flea control schedule	282	74.0
I am not aware of the deworming and flea control schedule	95	24.9

Table 4 (*continued*)

	Frequency (n)	Percentage (%)
Knowledge of the Purpose of Neutering[a]		
Decrease the overpopulation of strays	340	36.5
Decrease transmissible diseases	223	23.9
Decrease aggressiveness and inappropriate behaviour	212	22.7
Reduce chances of tumour	157	16.8
Concerns Pertaining to the Neutering Procedure[a]		
Pain	303	35.4
Wound complications	224	26.2
Pet's mood will change	71	16.6
Pet will become lazy or inactive	75	8.8
Pet will get fat	41	8.3
Unable to express normal behaviour	142	4.8

* [a] Question allows for more than one answer choice

Knowledge on the Frequency of Bringing Pets for Preventive Healthcare

In terms of awareness and knowledge on the suggested frequency of bringing their pets to the veterinary clinic for health check-ups, deworming, and flea control, the majority answered that the recommended visit is every three months (Table 5). In assessing the awareness of vaccination frequencies, the results showed a division in respondents' knowledge. While 48%(n=183) indicated a three-month interval, this may reflect a focus on the primary vaccination series required for young animals to complete their initial boosters. In contrast, the 30% (n=80) chose "other" as their answer, where the majority noted "once a year" or "annually" demonstrated awareness of long-term maintenance protocols for adult pets. This part measures theoretical knowledge of the required schedule rather that the owners' actual adherence or current practice.

Table 5
Knowledge on the recommended frequency of bringing pets for preventive healthcare

Variables	Type of Responses				
	No need N (%)	Once in a month N (%)	Once in 3 months N (%)	When sick N (%)	Other N (%)
How often should you bring your pets to the vet for a health check-up	3 (0.9%)	35 (10.0%)	195 (55.6%)	102 (29.1%)	16 (4.6%)
How often should you bring your pets to the vet for vaccination	7 (1.8%)	40 (10.5%)	183 (48.0%)	41 (10.8%)	80 (30.0%)
How often should you bring your pets to the vet for deworm	8 (2.3%)	69 (19.7%)	212 (60.4%)	38 (10.8%)	24 (6.8%)
How often should you bring your pets to the vet for deflea	9 (2.6%)	87 (24.8%)	193 (55.0%)	40 (11.4%)	22 (6.3%)

A multiple linear regression analysis was conducted to evaluate the influence of demographic factors on knowledge scores regarding neutering and preventive healthcare. Table 6 showed that age and education level were identified as significant predictors of knowledge ($P < 0.05$). The results indicated a positive correlation, suggesting that respondents within the 21-30 age group and those holding bachelor's degree demonstrated significantly higher knowledge scores compared to other demographic factors. This suggests that young adults with tertiary education are more informed regarding pet health requirements in Selangor.

Perception of Preventive Healthcare and Neutering

In general, the Selangor public showed a moderate perception on preventive healthcare and neutering [Low perception: 4 to 9; Moderate perception: 10 to 14; High perception: 15 to 20]. Using Likert-scale type questions, only 31% to 42% of respondents strongly agreed with statements regarding neutering or spaying. Despite the majority being cat owners, only 19.1% are strongly confident that they are knowledgeable about proper cat care (Table 7).

Table 6
Linear regression test on demographic variables and knowledge

	R-squared value	Unstandardised B	Significant (*P*-value)
Age		-0.190	0.339
Gender		0.236	0.554
Race		-0.119	0.596
Education Level	0.014	0.347	0.121
Profession		0.137	0.369
Household Income		0.148	0.638

Table 7
Perception of preventive healthcare and neutering

	Mean ± Standard Deviation (SD)	*P*-value
"Neutering cats or dogs can reduce the risk of infectious diseases between cats or dogs" [a]	4.02 ± 0.977	$P < 0.05$
"Cat or dog neuter can reduce the desire to mate" [a]	4.05 ± 0.988	$P < 0.05$
"Cats that are neutered will be more docile" [a]	3.83 ± 1.021	$P < 0.05$
"I am knowledgeable about cat care" [a]	3.55 ± 1.008	$P < 0.05$
"I am knowledgeable about dog care" [a]	2.47 ± 1.327	$P < 0.05$
"Cats can take care of themselves" [a]	3.20 ± 1.110	$P < 0.05$
"Dogs can take care of themselves" [a]	3.03 ± 1.146	$P < 0.05$
"A dog that is trained will be more docile" [a]	3.56 ± 1.254	$P < 0.05$
"Vaccination protect cats or dogs from contracting any diseases" [a]	4.14 ± 0.977	$P < 0.05$
"Having a cat or dog is expensive" [a]	3.48 ± 1.184	$P < 0.05$

*[a] Question allows for more than one answer choice

Practice regarding Healthcare and Neuter

Regarding the actual practice of bringing pets to the veterinary clinic for preventive healthcare, most pet owners revealed that they only seek professional help for health checkups when their pets are sick. As for the frequency of getting their pets dewormed, defleaed, and vaccinated, similar answers were seen as stated previously in the knowledge section, where the majority responded with a visit every three months. Table 8 shows the descriptive statistics on pet owners' practice of bringing their pets to the veterinary clinic for preventive healthcare.

Association between Knowledge, Perception, and Practice of Pet Owners regarding Neutering and Preventive Healthcare

To study the relationship between the knowledge, perception, and practice of the Selangor public regarding neutering and preventive healthcare, Spearman's correlation was carried out. As shown in Table 9, the correlation coefficient between all three variables yields a positive correlation (Spearman's rank correlation, $r_s = 0.665, 0.168, 0.150$, $P < 0.001$) with a P-value showing a statistically significant relationship between knowledge, attitude, and perception.

Education Intervention

Table 10 shows respondents' preferred method of educational intervention on preventive healthcare and neuter in the future. Most respondents chose veterinarians as their primary source of education regarding pet healthcare, followed by online sources such as Google (25.2%, n = 235), and social media platforms such as Facebook, Instagram, and TikTok (20.9%, n = 195).

Table 8
Pet owners' practice on preventive healthcare

Variables	Type of Responses				
	No need N (%)	Once in a month N (%)	Once in 3 months N (%)	When sick N (%)	Other N (%)
How often do you bring your pets to the vet for health check-up	22 (6.3%)	28 (6.8%)	97 (27.6%)	199 (56.7%)	9 (2.5%)
How often do you bring your pets to the vet for vaccination	29 (8.3%)	36 (10.3%)	123 (35.0%)	64 (18.2%)	99 (28.2%)
How often do you bring your pets to the vet for deworm	27 (7.7%)	64 (18.2%)	168 (47.8%)	52 (14.8%)	40 (11.4%)
How often do you bring your pets to the vet for flea control	28 (7.9%)	81 (23.1%)	152 (43.3%)	55 (15.4%)	35 (9.9%)

Table 9
Spearman's correlation test for knowledge, perception, and practice

Variables	Correlation Coefficient, r	*P*-value
Knowledge and Perception	0.168	0.001
Knowledge and Practice	0.665	<0.001
Perception and Practice	0.150	0.005

Table 10
Preferred educational intervention

	Frequency (n)	Percentage (%)
Education intervention [a]		
Veterinarian	275	29.5
Online sources (e.g., Google)	235	25.2
Friends and family	147	15.8
Pet care books, magazines, etc.	80	8.6
Social media platforms (e.g., Facebook, Instagram, TikTok, X, YouTube)	195	20.9

*[a] Question allows for more than one answer choice

DISCUSSION

This study demonstrates that the public's perspective and actions regarding the health and welfare of pets are still not on par with their knowledge levels. According 2023 study conducted in the United States, there has been a decrease in the frequency of veterinary visits compared to the previous years, which coincides with the increasing use of social media that hosts plenty of pet care information provided by unqualified personnel (AVMA, 2023; Evason, 2021). Thus, the present study specifically focuses on the residents of the most densely populated and racially diverse state in Malaysia, aiming to determine the public's current knowledge, perception and practices to inform effective educational intervention.

The current findings reveal that most respondents were female at nearly double the rate of males. These results are consistent with a study by Debbra et al. (2019) and Zaini et al. (2025 which noted that in Malaysia, females (64%) were more inclined to participate in survey-based research than males (36%) (Zaini et al., 2025). This questionnaire also garnered the most responses from participants aged 21 to 30 years old. While the survey was distributed both physically and online, the majority of responses came from the latter. This outcome aligns with findings by Mohamed et al. (2021), stating that the predominant age group among internet users in Malaysia is between 18 to 29 years old.

The findings of this study indicate that while the public possesses a moderate level of knowledge regarding pet preventive healthcare, this awareness is not reflected in their self-reported practice. For example, a majority of respondents stated that vaccinations are

administered every three months, which does not reflect the actual vaccination guidelines and protocol according to the World Small Animal Veterinary Association (WSAVA) guidelines (Squires et al., 2024). Core vaccinations for cats and dogs are typically administered monthly for the first three months, followed by annual boosters. Specifically, these core protocols protect dogs against canine distemper virus (CDV), canine adenovirus (CAV), and variants of canine parvovirus type 2 (CPV-2), while guarding cats against feline parvovirus (FPV), feline calicivirus (FCV), and feline herpesvirus-1 (FHV-1). This ensures that cats and dogs achieve optimal herd immunity, which is not only beneficial for themselves but also controls the transmission of infectious and highly communicable diseases within the community (Day et al., 2016).

In addition to vaccination awareness, respondents identified that "once a month" or "once every three months" as the typical frequency for parasite control, which aligns with the application instructions for most commercial products. According to the European Scientific Counsel Companion Animal Parasite (ESCCAP, 2021), adult cats and dogs are recommended to be dewormed against intestinal parasites such as roundworm, heartworms, tapeworms, hookworms, and whipworms at least once a month, specifically for pets with outdoor access or those that hunt prey. For strictly indoor pets, deworming every three months or four times a year is sufficient, with regular faecal examinations conducted to monitor for active infestation (ESCCAP, 2021). As for heartworm prevention, the American Heartworm Society (AHS, 2024) guidelines state parasiticide should be administered once a month as a protection from *Dirofilaria immitis* using FDA-approved active ingredients such as macrocyclic lactones (AHS, 2024).

It is noted that the statement *"bringing pets for a checkup at the veterinarian only when they are sick"* received the highest level of agreement among respondents. This highlights a significant gap in adherence to preventive healthcare guidelines devised by AAHA and AVMA (2020), which recommend annual health checkup aid in the early detection of underlying health diseases, especially in geriatric animals (Chowdhury, 2023). Comprehensive health screening, including taking blood chemistry profiles, diagnostic imaging, and oral health assessments are essential for identifying incidental findings such as neoplasia, diabetes mellitus, and periodontal diseases before they become clinically advanced.

Approximately 90% of respondents showed a good understanding of neutering, with the majority also exhibiting a moderate to high perception of the subject. This indicates that the Selangor population maintains a positive view of the ovariohysterectomy (spaying) and castration of cats and dogs, which aligns with a previous study conducted in Klang Valley, Selangor (Munir et.al., 2023). Low-cost neutering programmes such as "Potong-Royong™", alongside the dissemination of information via social media, have shown to be effective in educating the public about the importance of neutering not only

for their household pets, but also as a critical strategy for mitigating stray overpopulation and reducing the transmission of communicable diseases (Munir et.al., 2023).

An important issue identified in this study relates to pet owners' concerns regarding the neutering procedure. Most respondents stated that post-operative pain is of huge concern, influencing their decision to neuter. According to Gunaseelan et al. (2013), who noted that pet welfare concerns, specifically the perception of pain, play a more significant role in decision-making than the availability of low-cost services. As a result, veterinarians must employ more effective communication strategies to address these concerns, providing reassurance regarding modern analgesia to avoid misconceptions that can deter owners from adopting this practice.

The current findings also revealed that the most preferred method of educational intervention was through veterinarians, followed by online search engines and social media. In accordance with this, a more targeted approach would be more effective in educating the public on preventive healthcare and neutering for cats and dogs. Taking into example the situation in Singapore, the public exhibits a positive attitude on this matter, which is attributable to the education initiated by organisations in the country, as well as the government's efforts in educating the public on pets' wellbeing. According to a study by Gunaseelan et al. (2013), Theory of Planned Behaviour (TPB) was applied to illustrate the relationship on how knowledge can affect practice and perception, such as frequent visits to the veterinary clinic, routine vaccinations and deworming, and getting their pets neutered. The theory of human behaviour in this context also demonstrated that by increasing knowledge of the public by constantly providing information through credible sources, such as veterinarians during clinic visits, or online infographics on social media by reliable organisations such as the DVS or Malaysian Veterinary Medical Association (MAVMA), acts as a catalyst for improved practices. By addressing cognitive barriers and providing accessible information, pet owners are more likely to adopt proactive healthcare routines, including routine vaccination, deworming and neutering. This framework serves as a vital template for Malaysia to seek responsible pet ownership amidst a growing pet population.

LIMITATIONS AND RECOMMENDATIONS

A primary limitation of this study is the restricted sample size area and geographical scope, which may not fully represent the total population of Selangor. This limitation introduces potential bias and may undermine the generalizability of the findings. The majority of respondents consists of cat owners, which leads to over-representation of this group and under-representation of dog owners. Consequently, the findings may not accurately reflect the attitudes and practices related to dogs. Additionally, the data collection relied on convenience sampling via social media and localised physical distribution, specifically at the SPCA during the "Potong-Royong™" programme. This methodology

excluded pet owners without social media were not able to participate, which could have garnered a different outcome for this study. To enhance the validity for future research, it is recommended that participants be recruited from a broader range of districts across Selangor (i.e., urban areas and rural areas) to ensure a more representative sample. Future studies should also implement a stratified sampling approach to maintain a balanced ratio between cat and dog owners, thereby avoiding demographic skew. Then, translating the survey instruments into additional languages, such as Tamil and Mandarin, is encouraged to accommodate Malaysia's multicultural socioeconomic landscape and yield more comprehensive results.

CONCLUSION

In conclusion, the Selangor public showed a moderate level of knowledge and perception regarding neutering and preventive healthcare for the well-being of cats and dogs. However, a significant discrepancy was observed between participants' knowledge and their actual practice in pet care. This gap suggests that knowledge alone is insufficient to drive action, indicating that various internal and external factors influence the translation of awareness into behaviour. These findings highlight the necessity of incorporating psychological constructs- such as Human Behaviour Change Model- into future studies to better understand the mechanisms of human behavioural change. Thus, more extensive, evidence-based interventions delivered through targeted mediums are needed to emphasise the importance of preventive healthcare and ultimately enhance the welfare of cats and dogs in Selangor.

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Review Article

The Ecology and Conservation of Orchids in Ultramafic Habitats of Kinabalu Park, Sabah, Malaysia

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ABSTRACT

Kinabalu Park in Sabah, Malaysia, is renowned for its exceptional biodiversity, particularly its diverse orchid (Orchidaceae) flora, many of which grow in ultramafic soils. These soils are known to contain high levels of nickel and magnesium, low levels of calcium, few essential nutrients, and low water retention, hence bringing serious ecological problems to the survival of plants. This paper summarises existing information on the ecology and conservation of orchids in the ultramafic zone of Kinabalu Park, with emphasis on how the species adapt and why conservation efforts are necessary. This work is completely grounded on secondary data and is based on an in-depth evaluation of scientific literature (articles, books, journals, and online herbarium records) to evaluate the orchid diversity and ecological significance in the park. It has been found that there are up to 340 orchid species in the ultramafic sections of the Kinabalu Park, the highest density of which is Marai Parai (136 species), home to the locally endemic *Dendrobium maraiparense* Wood and Chan, described first at this location. Significant dangers such as excessive collection, illegal commerce, poaching, and climate change demonstrate the necessity of a more powerful conservation and rescue policy. The use of in-situ and ex-situ methods of conservation, increasing community education, and legal enforcement of the control of orchid species are required to protect the orchid species and preserve the ecological health of Kinabalu Park.

Keywords: Endemic, Mount Kinabalu, Marai Parai, UNESCO global geopark

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INTRODUCTION

Ultramafic outcrops are known to host exceptionally high levels of endemism in the flora, including, though not limited to, species with specialised adaptations such as hyperaccumulation of nickel or manganese. The ultramafic rocks are found in Malaysia, the Philippines and Indonesia in tropical

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countries of Southeast Asia (Figure 1). Nevertheless, the ecological features are under-researched despite the great value of the species group in conservation. Such rocks are composed of the upper mantle of the Earth and usually contain magnesium and iron and have low silicates (Estrada, 2021; Jakub Kierczak et al., 2020). The soils that are created on the basis of such bedrock have comparatively high trace metals such as nickel (Ni), chromium (Cr), and cobalt (Co) and have severe cation imbalances with excess magnesium (Mg) and low calcium (Ca) (van der Ent et al., 2019). A distinct type of vegetation with high endemism and short stature has evolved as a result of this unusual soil chemistry. The primary soil-edaphic factors frequently identified as influencing ultramafic ecology include nutrient deficiencies caused by low Ca (and high Mg), extremely low potassium (K) and phosphorus (P), and the potential phytotoxicity associated with elevated Ni and, in some cases, Co and Cr (van der Ent et al., 2018; van der Ent et al., 2019).

In Southeast Asia, a high level of geological and environmental heterogeneity is shown in Malaysia, Indonesia, and the Philippines (Figure 2). Ultramafic soils in South and Southeast Asia have a very diverse vegetation, from short graminoid communities in the upper montane and subalpine zones to tall lowland forests with tree heights exceeding 50 meters. At the landscape scale, the level of endemism is consistently high. For example, a 250 m^2 montane forest plot on Mount Tambuyukon in Sabah recorded 132 species, placing it among the most biodiverse plant communities globally (Garnica-Díaz et al., 2022; van der Ent et al., 2016).

The Malaysian state of Sabah is recognised as one of the most environmentally diverse regions in the world, with an estimated 8,000 species of vascular plant (van der Ent et al., 2015a). While 4,252 plant species from 207 families and 1,047 genera

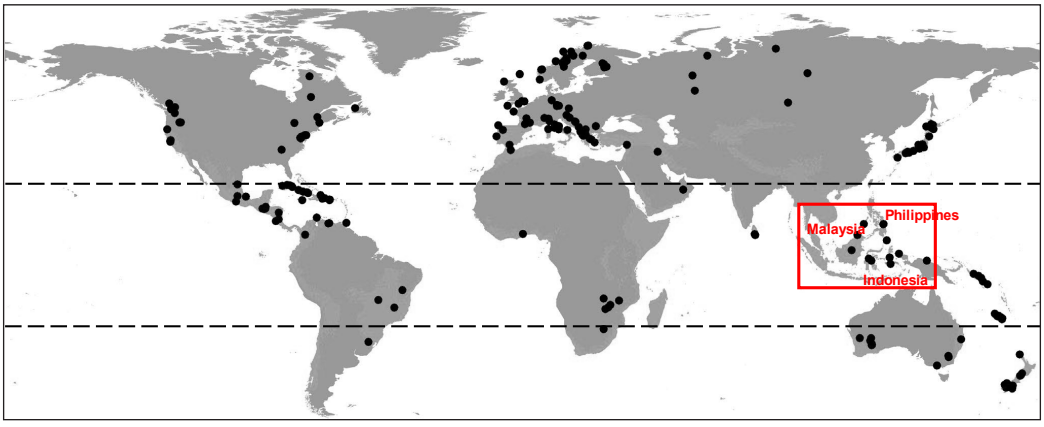

Figure 1. The distribution of ultramafic rocks worldwide. Black dotted - ultramafic formations are found on every continent; Red box - Southeast Asian countries where ultramafic rocks are distributed; Dashed lines - the Tropic of Cancer and Capricorn

Source: Modified from Hulshof and Spasojevic (2020)

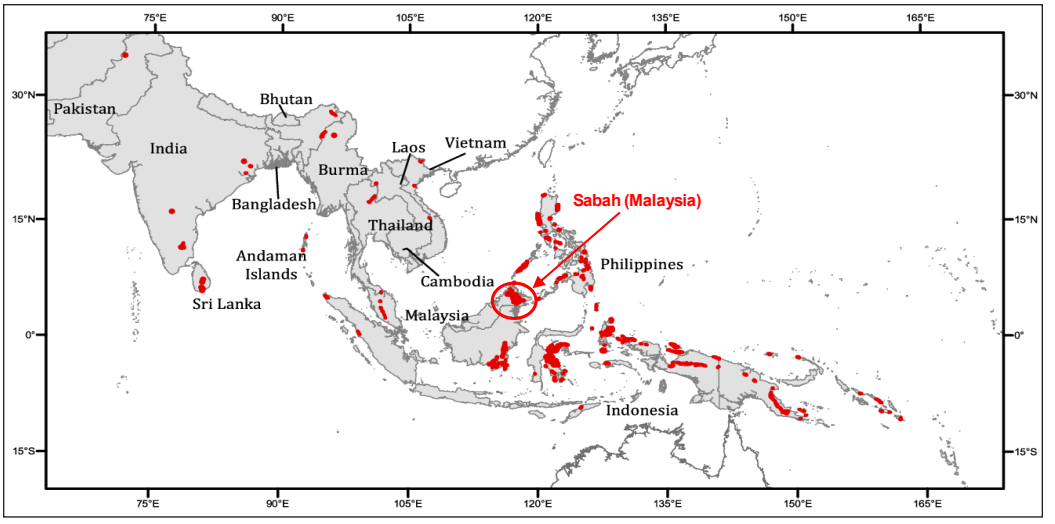

Figure 2. A close-up map of South and Southeast Asia displays the ultramafic outcrop spread throughout the area. Red circle - ultramafic outcrop spread throughout Sabah, Malaysia

Source: Modified from Galey et al. (2017)

(including vascular plants and ferns) were documented on ultramafic soils (Hulshof & Spasojevic, 2020; van der Ent et al., 2015a). Kinabalu Park, with an area of approximately 754 km^2, supports more than 5,000 plant species representing 200 families and 1,000 genera (Sabah Park, 2025). The park is one of the most biodiverse locations on Earth, as it has more than 2,542 species found in ultramafic outcrops. Most of these species are endemic to Kinabalu Park or Borneo Island, highlighting the uniqueness and extraordinary conservation importance of the flora.

Many of the most diverse plant groups in Kinabalu Park--including a stunning diversity of orchid species are found on serpentine-derived soils. Ultramafic substrates with unique chemical characteristics, namely serpentine, support highly specialised ecological communities. As an example, the high-magnesium and high-nickel toleration of some orchid species is exceptional (e.g. *Paphiopedilum rothschildianum* (Rchb.f.) Stein, *Crepidium metallicum* (Rchb.f.) Szlach, and *Dendrobium maraiparense* J.J. Wood & C.L. Chan), traits rarely observed in orchids from non-ultramafic environments. The majority of these species have slow growth rates, which could be one of the reasons why they become confined to edaphic habitats in which their fast-growing competitors effectively fringe them out.

This paper is the inaugural synthesis of ecological adaptation that incorporates diversity along with conservation concerns in the ultramafic community of Kinabalu Park, Sabah, Malaysia. Although the existing literature has recorded the floristic richness of the park, the little literature that has been conducted has not examined how the edificial factors that include soils, heavy-metal tolerance and orchid-mycorrhiza association all interact to

determine orchid distribution patterns. This paper can contribute to the study of diversity in orchids by providing a new view on the role of ultramafic habitat as an area of endemism and specialisation.

This work is grounded on the review of published scientific literature, herbarium records, and other secondary sources. There was no new fieldwork; rather, it is aimed to synthesise the available information to deepen the knowledge of species diversity, ecological adaptations, and species conservation concerns specific to orchids in ultramafic settings. Finally, the current review will guide conservation efforts in one of the most distinctive areas of Borneo on the botanical scale. It can also establish the basis of future studies by revealing important species of interest and pointing out existing gaps in knowledge.

ECOLOGICAL ASPECT OF ULTRAMAFIC IN SABAH

The ultramafic landscape is preserved and extensive in Sabah, Malaysia, which is one of the most significant areas in the globe to gain an insight into ultramafic studies. This region is full of endemism; soils are hostile with the extreme levels, and environmental stress is setting in. Kinabalu Park is home to one of the largest ultramafic ecosystems in Southeast Asia, where the ultramafic rock formations occupy up to 16% of the total vegetation cover (Sabah Park, 2025). The areas possess peculiar geological and ecological features.

The extremely harsh conditions are common to ultramafic soils that have a high degree of metal toxicity, low water-retention capacity, and nutrient deficiencies that have stimulated the development of highly specialised plant species (Galey et al., 2017). Many species can be found endemically in Sabah: orchids, pitcher plants (Nepenthes), and metal hyperaccumulator species like *Rinorea bengalensis*, which is found nowhere in Sabah or Borneo (van der Ent et al., 2015b). This situation can be studied to determine how plants have evolved to survive in extreme environments, and these areas include the variety of nutrient-acquisition methods, metal tolerance, and symbiotic relationships between plant roots and fungi.

The detailed ecological theories are also used to explain the distributions of orchid endemism and variation of the ultramafic habitat of the Kinabalu Park. Isolated ultramafic outcrops act as "edaphic islands" in the context of island biogeography, where limited soil conditions encourage high levels of endemism but limit species dispersal (Madsen et al., 2025). Orchid persistence on these substrates also reflects niche conservatism, with generations maintaining strong habitat fidelity and long-term adaptation to metal-bearing soils. Furthermore, because many orchid species exist in small, dispersed populations at risk of local extinction but are maintained by infrequent colonisation events, their distribution across the isolated ultramafic landscapes is consistent with metapopulation theory. Together, orchid diversity is shaped by the framework of soil specialisation, population dynamics and spatial isolation interacting in the ultramafic habitats of Kinabalu Park.

Plant Endemism

Soil edaphism greatly influences endemism in Sabah, making it an important global centre of plant endemism, particularly in its ultramafic ecosystems. Levels of endemism vary regionally among species inhabiting ultramafic substrates. Strong climatic and edaphic stressors, coupled with geographic isolation of many ultramafic outcrops, have likely facilitated speciation. However, most plant species in Sabah occur facultatively on ultramafic soils, and only a limited number are considered obligate ultramafic specialists. High endemism is usually in geographically or altitudinal remote areas. While some species exhibit broad ecological tolerances and occur across multiple substrates, others show a strong affinity to ultramafic soils and may be more abundant or entirely restricted to particular sites.

Neo-endemics are species that evolved *in-situ* from closely related taxa and often indicate recent speciation on ultramafic substrates. For example, *Dendrobium maraiparense* J.J. Wood & C.L. Chan, described from Marai Parai, is a neo-endemic confined to the ultramafic habitats of Kinabalu Park. In contrast, paleo-endemics are relict species now restricted to ultramafic soils due to competitive exclusion elsewhere; for instance, *Paphiopedilum rothschildianum* (Rchb. f.) Stein is a paleo-endemic frequently associated with ultramafic habitats. In this review, the term "ultramafic-associated" refers to species that have been consistently recorded on ultramafic soils based on floristic survey, herbarium data, and literature reports, rather than species with experimentally confirmed edaphic tolerance.

Orchids constitute an ecologically significant and highly diverse plant family in Sabah (Zhang et al, 2018), with substantial levels of endemism in ultramafic regions, especially within Kinabalu Park. Ultramafic obligate endemic orchids include both terrestrial and lithophytic slipper orchids: *Paphiopedilum rothschildianum* (Rchb. f.) Stein and *P. dayanum* (Rchb. f.) Pfitzer (Kinabalu Park), *P. hookerae* var. *volonteanum* (Sander ex Rolfe) Braem (Meliau Range, Kinabalu Park), the terrestrial *Calanthe otuhanica* C.L.Chan & T.J.Barkman (restricted to landslide zones in Kinabalu Park), *Platanthera kinabaluensis* Kraenzl. ex Rolfe and *Coelogyne rupicola* Carr (high-altitude ultramafic scrub), and the epiphytes *Paraphalaenopsis labukensis* Shim, A.Lamb & C.L.Chan, *Arachnis* longisepala (J.J.Wood) Shim & A.Lamb, and *Porpax borneensis* J.J.Wood & A.Lamb (limited accessibility in lowland ultramafic forests).

The endemism in orchids is a factor that is affected by both intrinsic and extrinsic factors in this environment. Examples of intrinsic factors are biological attributes affecting orchid reproduction and survival, like the use of mycorrhizal fungi to germinate seeds, reproductive isolation through being highly inbreeding-depressive, or reproductive isolation through limitation of pollination (Yang et al., 2021). Orchids are very sensitive to all environmental factors that have a direct impact on the population dynamics and reproductive success. Extrinsic factors are mainly manmade and commonly occur in combination with natural

environmental stressors. Habitat degradation and climate change influence the orchid distribution and ecological interaction, and biological pressures (pollinator decline, invasive species competition, etc.) contribute to the aggravation of conservation issues (Morales et al., 2017).

Mycorrhizal Associations

The microbial and fungal relationships are vital in the adaptation of plants to the ultramafic soils. Orchid plants are also mycorrhizal symbionts that can live in habitat characterised by severe soil conditions. These networks increase the resilience of ultramafic flora and are involved in the increased ecological role of these landscapes.

Mycorrhizal fungi, especially *Rhizoctonia* or *Tulasnella* genera, are needed by orchids specialised to ultramafic habitats (Arriagada et al., 2015). Mycorrhizal fungi have been associated with the acquisition of nutrients, particularly those of nitrogen and phosphorus and during early development of orchids, they are of great significance. They control the germination of seed and affect growth, which will determine survival, abundance, and spatial distribution (Yeh et al., 2019). Orchids have no endosperm in their orchid seeds, hence are entirely reliant on external sources of nutrients to germinate. When intoxicated into the orchid tissues, the fungi form platoons, and an exchange of nutrients takes place. The interaction is called mycoheterotrophy and enables orchids to acquire nutrients via fungal mediation and is at least partially mutualistic, because the fungi also obtain organic carbon through the orchid (Read et al., 2024).

This symbiosis is critical to endemic orchids like *Paphiopedilum rothschildianum* because the plants are only found in ultramafic soils of high altitude and are rich in metals within the Kinabalu Park. Without the help of fungi, orchids would fail to grow and to live in the inhospitable environment.

GLOBAL COMPARISON OF ULTRAMAFIC ORCHID DIVERSITY

Ultramafic habitats all around the globe are marked by ecological specialisation, great endemism, and adaptation of plants to soils rich in heavy metals. Having a high percentage of endemic species that are limited by edaphic factors and altitude, Kinabalu Park in Borneo is one of the richest centres of orchid diversity in the world, which is tied to ultramafic substrates. Other ultramafic parts of the Earth present a number of similarities and differences when compared to this region.

In New Caledonia, ultramafic soils cover roughly one-third of the island and support exceptional plant diversity. More than 3,309 species of ultramafic flora (2,492 of which are endemic) are highly specialised and endemic (Isnard et al., 2016). Even though orchid diversity is not as high as in Borneo, a number of genera can grow on nickel-containing materials, which suggests high adaptability to serpentine soils.

In Cuba, orchids form an important component of serpentine outcrops' floras, which function as hotspots of plant diversity and endemism. Habitat-driven distribution patterns and narrow-range endemism are comparable to Southeast Asia, although orchid richness there is lower. In California, the diversity of orchids in serpentine habitats is also lower than in tropical ultramafic regions, but orchids are suitable as valuable examples of chemically challenging soils. Despite differences in overall richness, the presence of edaphic-specific features in some temperate species suggests that orchids across climatic zones may have evolved similar ecological strategies.

Table 1 summarises selected South and Southeast Asian countries with ultramafic floras and the number of ultramafic endemics. This global comparison highlights the uniqueness of the ultramafic environment of Kinabalu Park as a potential tropical centre for orchid diversity. Kinabalu Park contains hundreds of species, many of which are endemic, compared to California, which supports only a few orchid taxa. Kinabalu orchids exhibit a unique combination of strong land specialisation, high species richness, and significant conservation importance compared to regions such as New Caledonia and Cuba. This highlights the global importance of Kinabalu Park as an orchid biodiversity hotspot and reinforces its status as a conservation priority.

ORCHIDS IN ULTRAMAFIC HABITATS OF SABAH

Sabah is known for its extensive ultramafic landscape. Ultramafic rocks underlie approximately 3,500 km^2 of the state, representing about ~4.6% of its total land area.

Table 1
Comparison of orchid diversity and endemism across selected ultramafic landscapes worldwide

Region	Ultramafic Coverage (km2)	Total Number of Vascular Plant Species in the Flora	Number of Ultramafic-associated Species	Number of Ultramafic Endemic Species (% Ultramafic Endemism)	Orchid Richness	References
Kinabalu Park (Borneo)	754 square km	5000	-	2542 (50.8%)	High (hundreds of spp.)	Sabah Park, 2025
New Caledonia	1/3 of the island	3309	2492	1178 (47.3%)	Moderate	Isnard et al., 2016
Cuba	7% of the land area	6375	-	920 (14.4%)	Moderate	Reeves et al., 1999
California (USA)	~6,360 km² (<1.5% of 423,970 km²)	Not specified precisely; >1315 endemic species	255 taxa (strict + broad endemics)	14.7% of endemic species	Low	Safford & Miller, 2020

As shown in Figure 3, the largest ultramafic outcrops extend from Mount Tawai, Meliau Range, and the Bidu-bidu Hills through Mount Silam to Mount Kinabalu. Specific plant species found in Sabah's ultramafic soils have adapted to environments with high concentrations of heavy metals and low nutrient availability (Meindl et al., 2021). Consequently, these ultramafic areas are of exceptional ecological and botanical significance. The distribution of these rocks spans a broad elevational range, from low-lying islands in Darvel Bay to nearly 2,900 m above sea level on Mount Kinabalu.

The Sabah ultramafic flora is remarkably species-rich in contrast to other better-known ultramafic areas, e.g., New Caledonia, California (USA), Queensland (Australia), and Zimbabwe. The soil sustains very diverse vegetation, and the orchid is especially endemic. The number of orchid species is approximately 1,300 in Sabah, with approximately 250 species being endemic. This diversity is brought about mostly by the fact that they exist at Mount Kinabalu, the tallest mountain on the Malay Archipelago. Its varied ecosystem, comprising lowland rainforests to alpine meadows offer habitats where different assemblages of orchids thrive (Md.-Isa et al., 2024). Orchids can acclimatise to high levels of heavy metals by a symbiotic mutualism with fungi and specialised root systems.

Supplementary 1 shows that 563 orchid species have been recorded in Mount Kinabalu and other ultramafic outcrops in the state of Sabah. These data were assembled by looking at books, journals, articles, and herbarium database, but screened so as to obtain only those records in which the locality is known exactly; records which were simply labelled Sabah or those in cultivated plantations were ignored. Amongst the notable species, there are *Campanulorchis leiophylla* (Lindl.) Y.P.Ng & P.J.Cribb, a highly localised and

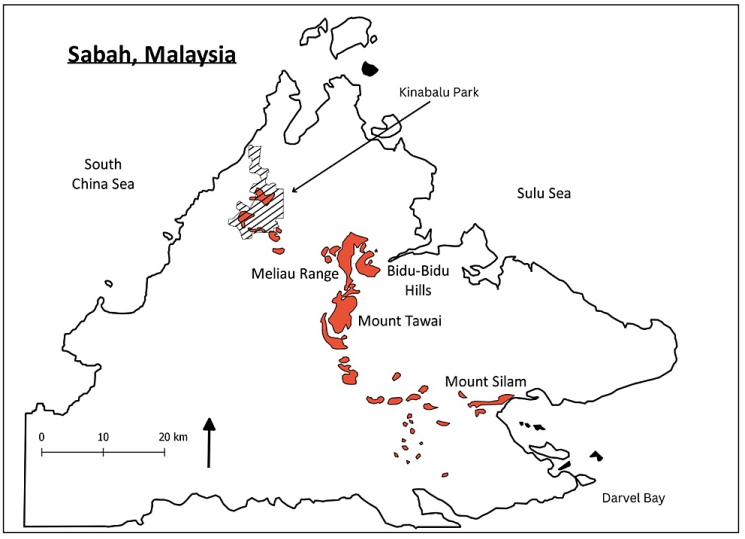

Figure 3. Igneous Rocks of Sabah, Malaysia. The line pattern fill - Kinabalu Park is situated
Source: Modified from van der Ent et al. (2011)

taxonomically unique orchid; *Bulbophyllum longhutense* J.J.Sm., endemic to Borneo and restricted to montane forests; and *Ascideria longifolia* (Hook.f.) Seidenf., a monopodial orchid with a unique taxonomy; all three demonstrate the importance of Mount Kinabalu as a biodiversity hotspot. The survival of the species shows that the ecological processes, such as specialist pollination, are preserved. Orchids are extremely sensitive to changes in their environment, and the fact that they are found in abundant numbers on Mount Kinabalu is a testimony to the relatively undisturbed and well-preserved habitats.

In the ultramafic areas around the range of Mount Kinabalu, there is also less orchid species diversity: it has 31 species on Mount Tawai, 13 on the Meliau Range, seven on Mount Silam and just five species on the Bidu-bidu Hills, the least orchid diversity that is ever reported within the ultramafic sites of Sabah. Species such as *Ascidieria grandis* (Ridl.) J.J.Wood and *Bulbophyllum chanii* J.J.Verm, & A.Lamb occur in the Meliau Range, which are reflections of ecological niches that enable the adaptation to ultramafic soils. Epiphytic orchid *B. chanii* is reliant on dense forest cover and is highly humid throughout to maintain its climate conditions, conditions preserved through the Meliau Range and also crucial to orchid species that adapt to shady habitats to microclimatic change.

There are a lot of species that are confined to localities. As an example, the number of species documented in Bidu-bidu Hills is only four species, with few trees and/or hydration causing low humidity and limited populations of mycorrhizal fungi that may have been encountered. Species richness can also be underestimated due to limited botanical surveys. This orchid faces threats of loss of its habitat, climate change and other human-driven forces. Therefore, immediate conservation action is required to take care of these delicate ecosystems and their rare orchid biodiversity.

STATUS OF ORCHID IN ULTRAMAFIC AREA OF KINABALU PARK, SABAH

There are a few key locations in the largest ultramafic outcrops of the Kinabalu Park: i. Mount Nambuyukon, ii. Mount Tambuyukon, iii. Wuluh River, iv. Nalumad, v. Panataran valley, vi. Marai Parai, vii. Layang-layang, viii. Mesilau, and ix. Bukit Babi (van der Ent & Wood, 2013). Outside the park, the most extensive serpentinite outcrops are found in the Hampuan Hill Forest Reserve, Kulung Hill, and the Lohan Valley on the southern slopes of Mount Kinabalu, with smaller occurrences reported from Bambangan. Such ecosystems are usually characterised by bodies of serpentinite that are abraded by rivers to form small valleys that have steep sides. These sceneries usually contain a lot of landslides having different successional stages which slowly progress into a closed-canopy forest.

In Kinabalu Park, the ultramafic soils occur in 13 places that are mainly or wholly underlying (Figure 4). These locations demonstrate the importance of soils in diversifying plants and the phenomenal concentration of plant species in the ultramafic environments. The park is surrounded by ultramafic rock formations that occupy an area of about 151 km^2

around the granite massif of Mount Kinabalu (van der Ent et al., 2016). This form features Mount Tambuyukon, which is contained within the whole composition of ultramafic rocks. The largest outcrops are found at Mount Tambuyukon (90 km^2) and Panataran Valley (30 km^2) (van der Ent et al., 2013).

Kinabalu Park, characterised by rocky, nutrient-poor soils, support orchids that have evolved distinct adaptations across different localities. The abundance of both terrestrial and epiphytic orchids likely reflects the open forest structure typical of serpentinite soils. High endemism among terrestrial orchids may be linked to the physiological adaptations to adverse soil chemistry, while epiphytic orchids exhibit parallel adaptations to ultramafic conditions.

Because records of orchid species are incomplete or unavailable records in some areas, only the green-shaded regions in Figure 4 were included in this study. These regions represent sites with detailed species-level records documented in publications and herbarium collections, allowing a more accurate assessment of diversity. An estimated 340 orchid species have been recorded across ten ultramafic outcrops in Kinabalu Park: Mount Tambuyukon, Panataran Valley, Marai Parai, Bukit Babi, Bambangan, Nalumad, Mesilau, Layang-layang, Hampuan Hill, and Lohan Valley (Table 2). Records were included only when precise locality information was available; generic records lacking detailed site descriptions were excluded from the analysis.

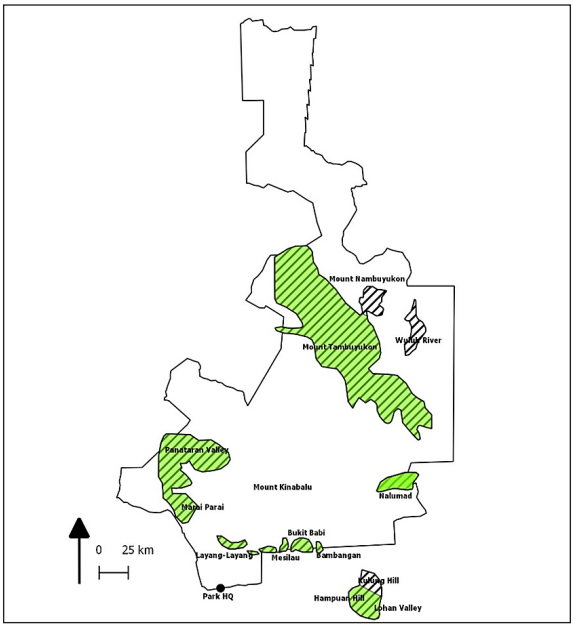

Figure 4. An overview of the major ultramafic outcrops in Kinabalu Park. The line pattern fill - regions indicate areas with ultramafic soils. These green-shaded areas represent locations where detailed records of orchid species have been found

Source: Modified from: van der Ent et al. (2015a)

Table 2
List of orchids species recorded in the ultramafic area of Kinabalu Park

Species	Ultramafic Areas in Kinabalu Park									
	Mount Tambuyukon[3,6,7,10,11]	**Panataran Valley**[3,7,8,11]	**Marai Parai**[3,4,7,8,9,10,11,12,13,14,15,18]	**Bukit Babi**[3]	**Bambangan**[3,8,11]	**Nalumad**[7,8,10,11]	**Mesilau**[3,4,11,12,13,14]	**Layang-Layang**[3,11,14]	**Hampuan Hill**[3,4,7,11,13]	**Lohan Valley**[3,4,7,8,11,15]
Appendicula congesta Ridl.	✓		✓							
Appendicula linearifolia Ames & C.Schweinf.			✓							
Appendicula longirostrata Ames & C.Schweinf.			✓							
Appendicula magnibracteata Ames & C.Schwein.			✓							
Appendicula fractiflexa J.J.Wood							✓			
Appendicula foliosa Ridl. & Schltr.							✓			
Appendicula lucida Ridl.										✓
Appendicula divaricata Ames & C.Schweinf.									✓	✓
Appendicula floribunda (Schltr.) Schltr.										✓
Appendicula pendula Blume		✓	✓		✓					
Appendicula reflexa Blume									✓	
Appendicula torta Blume					✓				✓	
Appendicula undulata Blume						✓				
Arachnis breviscapa (J.J.Sm.) J.J.Sm.										✓
Arachnis flos-aeris (L.) Rchb.f.						✓				
Arachnis longisepala (J.J.Wood) Shim & A.Lamb	✓								✓	✓
Arachnis calcarata Holttum			✓							✓
Arundina graminifolia (D.Don) Hochr.			✓			✓			✓	
Appendicula tembuyukenensis J.J.Wood	✓									
Appendicula anceps Blume			✓							
Ascidieria grandis (Ridl.) J.J.Wood						✓	✓			
Ascidieria cymbidiifolia (Ridl.) W.Suarez & Cootes			✓				✓			
Ascidieria maculiflora J.J.Wood	✓									
Ascidieria cymbidiifolia var. *pandanifolia* J.J.Wood	✓									
Ascidieria longifolia (Hook.f.) Seidenf.	✓									
Anoectochilus geniculatus Ridl.	✓									
Agrostophyllum globigerum Ames & C.Schweinf.			✓							
Agrostophyllum majus Hook.f.			✓							
Apostasia nuda R.Br.			✓							
Apostasia wallichii R.Br.	✓								✓	
Aeridostachya robusta (Blume) F.G.Brieger	✓		✓							
Adenoncos parviflora Ridl.										✓
Adenoncos borneensis Schltr.										✓

Table 2 (*continued*)

Species	Ultramafic Areas in Kinabalu Park								
	Mount Tambuyukon[3,6,7,10,11]	Panataran Valley[3,7,8,11]	Marai Parai[3,4,7,8,9,10,11,12,13,14,15,18]	Bukit Babi[3]	Bambangan[3,8,11]	Nalumad[7,8,10,11]	Mesilau[3,4,11,12,13,14]	Layang-Layang[3,11,14]	Hampuan Hill[3,4,7,11,13]	Lohan Valley[3,4,7,8,11,15]
Bromheadia longifolia Kruiz. & de Vogel			✓							
Bromheadia lohaniensis Kruiz. & de Vogel										✓
Bromheadia divaricata Ames & C.Schweinf.			✓							
Bulbophyllum apheles J.J.Verm.							✓			
Bulbophyllum gibbsiae Rolfe			✓							
Bulbophyllum mandibulare Rchb. f.										✓
Bulbophyllum gamosepalum (Griff.) J.J.Sm.							✓			
Bulbophyllum gibbosum (Blume) Lindl.							✓			
Bulbophyllum microglossum Ridley							✓			
Bulbophyllum apodum Hook.f.			✓			✓				
Bulbophyllum biflorum Teijsm. & Binn.										✓
Bulbophyllum breviflorum Ridl.							✓			
Bulbophyllum chanii J.J.Verm. & A.Lamb						✓				
Bulbophyllum coriaceum Ridl.			✓					✓		
Bulbophyllum dracunculus J.J.Verm.			✓							
Bulbophyllum dryas Ridl.		✓								
Bulbophyllum lambii J.J.Verm.							✓			
Bulbophyllum praetervisum J.J.Verm.										✓
Bulbophyllum sigmoideum Ames & C.Schweinf.							✓			
Bulbophyllum tortuosum (Blume) Lindl.									✓	
Bulbophyllum trifolium Ridl.									✓	
Bulbophyllum vaginatum (Lindl.) Rchb.f.						✓				
Bulbophyllum planibulbe Ridl.									✓	✓
Bulbophyllum depressum King & Pantl.							✓			✓
Bulbophyllum flavescens (Blume) Lindl.			✓		✓		✓			
Bulbophyllum vinaceum Ames & C.Schweinf.			✓							
Bulbophyllum anguliferum Ames & C.Schweinf.							✓			
Bulbophyllum aeolium Ridl.							✓			
Bulbophyllum caudatisepalum Ames & C.Schweinf.					✓		✓			
Bulbophyllum flammuliferum Ridl.									✓	
Bulbophyllum lobbii subsp. *lobbii*					✓		✓			✓
Bulbophyllum lobbii subsp. *boreoborneense* Mangal, F.Velazquez & J.J.Verm.							✓			
Bulbophyllum nubinatum J.J.Verm.	✓		✓							

Table 2 (*continued*)

Species	Ultramafic Areas in Kinabalu Park									
	Mount Tambuyukon[3,6,7,10,11]	Panataran Valley[3,7,8,11]	Marai Parai[3,4,7,8,9,10,11,12,13,14,15,18]	Bukit Babi[3]	Bambangan[3,8,11]	Nalumad[7,8,10,11]	Mesilau[3,4,11,12,13,14]	Layang-Layang[3,11,14]	Hampuan Hill[3,4,7,11,13]	Lohan Valley[3,4,7,8,11,15]
Bulbophyllum coniferum Ridl.	✓				✓					
Bulbophyllum disjunctum Ames & C.Schweinf.	✓		✓				✓			
Bulbophyllum deltoideum Ames & C.Schweinf.			✓				✓			
Bulbophyllum latisepalum Ridl. & C.Schweinf.							✓			
Bulbophyllum turgidum J.J.Verm.							✓			
Bulbophyllum retrorsum J.J.Verm. & A.Lamb							✓			
Bulbophyllum membranifolium Hook.f.			✓							
Bulbophyllum montense Ridl.			✓				✓			
Dendrochilum exasperatum Ridl.			✓							
Dendrochilum lancilabium Ridl.			✓							
Dendrochilum alatum Ridl.			✓							
Dendrochilum haslamii Ridl.							✓			
Dendrochilum joclemensii Ridl.			✓							
Bulbophyllum hyalosemoides J.J.Verm. & P.O'Byrne	✓						✓			
Bulbophyllum obtusum (Blume) Lindl.							✓			
Bulbophyllum unguiculatum Rchb.f.			✓				✓			
Bulbophyllum mutabile (Blume) Lindl.			✓							
Bulbophyllum pocillum J.J.Verm.			✓							
Coelogyne latiloba de Vogel			✓							
Coelogyne chanii Gravend. & de Vogel						✓				
Coelogyne craticulilabris Carr					✓					
Coelogyne pandurata Lindl.									✓	✓
Coelogyne planiscapa var. *planiscapa*			✓		✓					
Coelogyne septemcostata J.J.Sm.		✓								
Cleisocentron abasii Cavestro			✓							
Calanthe lyroglossa Rchb.f.			✓							
Calanthe woodii P.J.Cribb								✓		
Calanthe otuhanica C.L.Chan & T.J.Barkman	✓									
Calanthe musa-amanii J.J.Wood			✓							
Calanthe kinabaluensis Rolfe					✓					
Calanthe triplicata (Willemet) Ames					✓	✓	✓			
Calanthe vestita Wall. *ex* Lindl.									✓	
Calanthe lambii P.J.Cribb			✓				✓			
Callostylis pulchella (Lindl.) S.C.Chen & Z.H.Tsi						✓				

Table 2 (*continued*)

Species	Ultramafic Areas in Kinabalu Park									
	Mount Tambuyukon[3,6,7,10,11]	Panataran Valley[3,7,8,11]	Marai Parai[3,4,7,8,9,10,11,12,13,14,15,18]	Bukit Babi[3]	Bambangan[3,8,11]	Nalumad[7,8,10,11]	Mesilau[3,4,11,12,13,14]	Layang-Layang[3,11,14]	Hampuan Hill[3,4,7,11,13]	Lohan Valley[3,4,7,8,11,15]
Campanulorchis leiophylla (Lindl.) Y.P.Ng & P.J.Cribb			✓							
Ceratostylis longisegmenta Ames & C.Schweinf.							✓			
Ceratostylis pendula Hook.f.										✓
Ceratostylis ampullacea Kraenzl.	✓									
Cleisocentron merrillianum (Ridl.) Christenson	✓		✓							
Ceratostylis crassilingua Ames & C.Schweinf.			✓							
Chelonistele amplissima (Ames & C.Schweinf.) Carr							✓			✓
Chelonistele kinabaluensis (Rolfe) de Vogel							✓			
Chamaeanthus brachystachys Schltr.	✓								✓	
Chroniochilus minimus (Blume) J.J.Sm.	✓								✓	
Coelogyne radioferens Ames & C.Schweinf.				✓	✓		✓			
Coelogyne genuflexa Ames & C.Schweinf.			✓							
Coelogyne clemensii Ames & C.Schweinf.			✓				✓			
Coelogyne compressicaulis Ames & C.Schweinf.			✓							
Coelogyne kinabaluensis Ames & C.Schweinf.			✓		✓					✓
Coelogyne tenompokensis Carr							✓			
Coelogyne monilirachis Carr							✓			
Coelogyne swaniana Rolfe							✓			✓
Coelogyne exalata Ridl.							✓			
Coelogyne cuprea H.Wendl. & Kraenzl. var. *cuprea*	✓						✓			
Coelogyne cuprea var. *planiscapa* J.J.Wood & C.L.Chan	✓									
Coelogyne papillosa Ridl.	✓		✓				✓			
Coelogyne plicatissima Ames & C.Schweinf.	✓		✓		✓			✓		
Coelogyne hirtella J.J.Sm.			✓		✓		✓			
Coelogyne rupicola Carr			✓				✓			
Chroniochilus virescens (Ridl.) Holttum	✓									
Cystorchis aphylla Ridl.			✓						✓	
Cymbidium angustifolium Ames & C.Schweinf.			✓							
Cymbidium elongatum J.J.Wood, Du Puy & Shim	✓	✓	✓							
Cymbidium ensifolium (L.) Sw.							✓		✓	
Cymbidium sigmoideum J.J.Sm.	✓									
Cryptostylis acutata J.J.Sm.	✓		✓						✓	
Corybas pictus (Blume) Rchb.f.	✓		✓							
Cylindrolobus linearifolius (Merr.) J.J.Wood					✓					✓

Table 2 (*continued*)

Species	Ultramafic Areas in Kinabalu Park									
	Mount Tambuyukon[3,6,7,10,11]	Panataran Valley[3,7,8,11]	Marai Parai[3,4,7,8,9,10,11,12,13,14,15,18]	Bukit Babi[3]	Bambangan[3,8,11]	Nalumad[7,8,10,11]	Mesilau[3,4,11,12,13,14]	Layang-Layang[3,11,14]	Hampuan Hill[3,4,7,11,13]	Lohan Valley[3,4,7,8,11,15]
Cylindrolobus nutans (Lindl.) J.J.Wood		✓								
Cylindrolobus jensenianus (J.J.Sm.) Rauschert	✓									
Cleisocentron merrillianum (Ames) Christenson			✓							
Cleisostoma discolor Lindl.									✓	✓
Cleisostoma striatum (Rchb.f.) N.E.Br.						✓			✓	
Cleisostoma koeteiense (Schltr.) Garay										✓
Cleisocentron abasii Cavestro	✓		✓							
Crepidium metallicum (Rchb.f.) Szlach.	✓								✓	✓
Crepidium lowii (E.Morren) Szlach.									✓	✓
Crepidium mieczyslawii Marg. & Szlach.			✓							
Crepidium kinabaluense (Rolfe) Szlach.			✓				✓			
Dimorphorchis lowii (Lindl.) Rolfe	✓									
Dimorphorchis rossii Fowlie										✓
Dendrobium aloifolium (Blume) Rchb.f.										✓
Dendrobium longirepens Ames & C. Schweinfurth			✓							
Dendrochilum alatum Ames			✓							
Dendrobium anosmum Lindl.										✓
Dendrobium beamanianum J.J.Wood & A.Lamb			✓							
Dendrobium compressum Lindl.						✓				✓
Dendrobium gracile (Blume) Lindl.			✓				✓			
Dendrobium leonis (Lindl.) Rchb.f.									✓	✓
Dendrobium macrophyllum A.Rich.									✓	
Dendrobium pachyphyllum (Kuntze) Bakh.f.									✓	✓
Dendrobium panduriferum Hook.f.										✓
Dendrobium xanthoacron Schltr.							✓			
Dendrobium acuiferum Ormerod								✓		
Dipodium pictum (Lindl.) Rchb.f.				✓						
Dendrobium tetrachromum Rchb.f.			✓							
Dendrobium lambii J.J.Wood			✓							
Dendrobium daimandaui J.J.Wood						✓				
Dendrobium hirsutifolium J.J.Wood										✓
Dendrobium serena-alexianum J.J.Wood & A.Lamb	✓									
Dendrobium angustipetalum J.J.Sm.			✓							
Dendrobium alabense J.J.Wood			✓				✓			

Table 2 (*continued*)

Species	Ultramafic Areas in Kinabalu Park									
	Mount Tambuyukon[3,6,7,10,11]	Panataran Valley[3,7,8,11]	Marai Parai[3,4,7,8,9,10,11,12,13,14,15,18]	Bukit Babi[3]	Bambangan[3,8,11]	Nalumad[7,8,10,11]	Mesilau[3,4,11,12,13,14]	Layang-Layang[3,11,14]	Hampuan Hill[3,4,7,11,13]	Lohan Valley[3,4,7,8,11,15]
Dendrobium prostratum Ridl.									✓	✓
Dendrobium hirsutifolium J.J.Wood										✓
Dendrobium kiauense Ames & C.Schweinf.									✓	
Dendrobium patentilobum Ames & C.Schweinf.	✓		✓							✓
Dendrobium parthenium Rchb.f.			✓						✓	✓
Dendrobium olivaceum J.J.Sm.	✓									
Dendrobium cymbulipes J.J.Sm.	✓		✓		✓		✓			
Dendrobium minimum Ames & C.Schweinf.			✓							
Dendrobium spectatissimum Rchb.f.	✓									
Dendrobium kinabaluense Ridl.	✓									
Dendrobium tridentatum Ames & C.Schweinf.	✓		✓					✓		
Dendrochilum kinabuluense Rolfe			✓							
Dendrochilum simplex J.J.Sm.			✓							
Dendrochilum longifolium Rchb.f.			✓							✓
Dendrochilum anomalum Carr									✓	
Dendrochilum dewindtianum W.W.Sm.			✓					✓		
Dendrochilum imbricatum Ames					✓					
Dendrochilum longirachis Ames			✓							
Dendrochilum planiscapum Carr			✓							
Dendrochilum pterogyne Carr			✓					✓		
Dendrochilum fimbriatum Ridl.			✓							
Dendrochilum angustifolium Ridl.	✓		✓							
Dendrochilum crassilabium J.J.Wood	✓									
Dendrochilum corrugatum (Ridl.) J.J.Sm.			✓							
Dendrochilum cruciforme J.J.Wood var. *cruciforme*	✓									
Dendrochilum gibbsiae Rolfe	✓		✓							
Dendrochilum grandiflorum (Ridl.) J.J.Sm.	✓		✓							
Dendrochilum pallide-flavens Blume var. *pallidiflavens*			✓							
Dendrochilum kamborangense Ridl.	✓						✓			
Dendrochilum stachyodes (Ridl.) J.J.Sm.			✓					✓		
Dendrobium maraiparense J.J.Wood & C.L.Chan			✓							
Dendrochilum transversum Carr			✓							
Dendrobium piranha C.L.Chan & P.J.Cribb	✓		✓							
Dendrobium lancilabium J.J.Sm.	✓									

Table 2 (*continued*)

Species	Ultramafic Areas in Kinabalu Park									
	Mount Tambuyukon[3,6,7,10,11]	Panataran Valley[3,7,8,11]	Marai Parai[3,4,7,8,9,10,11,12,13,14,15,18]	Bukit Babi[3]	Bambangan[3,8,11]	Nalumad[7,8,10,11]	Mesilau[3,4,11,12,13,14]	Layang-Layang[3,11,14]	Hampuan Hill[3,4,7,11,13]	Lohan Valley[3,4,7,8,11,15]
Dendrobium lohanense J.J.Wood										✓
Dendrobium spurium (Blume) J.J.Sm.									✓	✓
Dendrobium rosellum Ridl.										✓
Dilochia parviflora J.J.Sm.			✓							
Dilochia cantleyi (Hook.f.) Ridl.	✓		✓				✓			
Dilochia wallichii Lindl.							✓			
Dilochia beamanii Ormerod							✓			
Dilochia gracilis (Ames & C.Schweinf.) Carr			✓							
Dendrobium bifarium Lindl. Ex Hook.f.									✓	
Neuwiedia borneensis de Vogel	✓								✓	
Bromheadia brevifolia Ridl.										✓
Bromheadia acerate Ames & C.Schweinf.	✓		✓							
Eria major Stapf		✓								
Eria villosissima Rolfe			✓							
Entomophobia kinabaluensis (Ridl.) de Vogel			✓							
Epigeneium tricallosum (Ames & C.Schweinf.) J.J.Wood							✓			
Epigeneium longirepens (Ames & C.Schweinf.) Seidenf.			✓							
Epigeneium kinabaluense (Ridl.) Summerh.	✓		✓	✓						
Epigeneium suberectum (Ridl.) Summerh.			✓							
Epidendrum longirepens (C.Schweinf.) C.Schweinf.	✓									
Eria javanica (Sw.) Blume			✓							
Eria linearifolia Ridl.										✓
Eria robusta (Blume) Lindl.							✓			✓
Eria oblitterata (Blume) Rchb.f.							✓			
Eria acerate Ames & C.Schweinf.							✓			
Eria aceratedes (Blume) Lindl.							✓			
Eria major Ridl. Ex Stapf							✓			
Eulophia graminea Lindl.										✓
Flickingeria scopa (Lindl.) Brieger										✓
Flickingeria fimbriata (Blume) A.D.Hawkes						✓				✓
Flickingeria xantholeuca (Rchb.f.) A.D.Hawkes						✓				
Gastrodia javanica (Blume) Lindl.						✓				
Geodorum densiflorum (Lam.) Schltr.									✓	
Goodyera bifida (Blume) Blume							✓			

Table 2 (*continued*)

Species	Ultramafic Areas in Kinabalu Park								
	Mount Tambuyukon[3,6,7,10,11]	Panataran Valley[3,7,8,11]	Marai Parai[3,4,7,8,9,10,11,12,13,14,15,18]	Bukit Babi[3]	Bambangan[3,8,11]	Nalumad[7,8,10,11]	Mesilau[3,4,11,12,13,14]	Layang-Layang[3,11,14]	Hampuan Hill[3,4,7,11,13]	Lohan Valley[3,4,7,8,11,15]
Goodyera condensata Ormerod & J.J.Wood							✓			
Goodyera rubicunda (Blume) Lindl.					✓					
Goodyera rostellata Ames & C.Schweinf.			✓				✓			
Habenaria muricata (Schauer) Rchb.f.						✓				
Hetaeria anomala Lindl.							✓			
Liparis kinabaluensis J.J.Wood										✓
Liparis viridicallus Holttum			✓						✓	
Liparis nervosa (Thunb.) Lindl.			✓							
Liparis elegans Lindl.										✓
Liparis pandurata Ridl.							✓			
Liparis kamborangensis Ames & C.Schweinf.	✓		✓							
Liparis atrosanguinea Ridl.			✓							
Lepidogyne longifolia (Blume) Blume			✓							
Malleola punctata J.J.Wood & A.Lamb									✓	✓
Micropera callosa (Blume) Garay									✓	
Micropera fuscolutea (Lindl.) Garay										✓
Mycaranthes latifolia Blume										✓
Mycaranthes magnicallosa (Ames & C.Schweinf.) J.J.Wood					✓					
Mycaranthes major (Stapf) J.J.Wood	✓		✓							
Neuwiedia borneensis de Vogel									✓	
Neuwiedia veratrifolia Blume									✓	
Neuwiedia zolingeri Rchb.f.									✓	
Nephelaphyllum flabellatum Ames & C.Schweinf.			✓						✓	
Nephelaphyllum pulchrum Blume							✓			
Nabaluia clemensii Ridl.							✓			
Nabaluia angustifolia de Vogel							✓			
Neuwiedia borneensis de Vogel	✓									
Neuwiedia zolingeri var. *javanica* (J.J.Sm.) de Vogel	✓									
Paphiopedilum lowii (Lindl.) Stein							✓			
Paphiopedilum rothschildianum (Rchb.f.) Stein	✓									
Phalaenopsis modesta J.J.Sm.										✓
Paraphalaenopsis labukensis Shim, A.Lamb & C.L.Chan	✓									
Phaius subtrilobus Ames & C.Schweinf.							✓			

Table 2 (*continued*)

Species	Ultramafic Areas in Kinabalu Park									
	Mount Tambuyukon[3,6,7,10,11]	Panataran Valley[3,7,8,11]	Marai Parai[3,4,7,8,9,10,11,12,13,14,15,18]	Bukit Babi[3]	Bambangan[3,8,11]	Nalumad[7,8,10,11]	Mesilau[3,4,11,12,13,14]	Layang-Layang[3,11,14]	Hampuan Hill[3,4,7,11,13]	Lohan Valley[3,4,7,8,11,15]
Phaius pauciflorus subsp. *Sabahensis* J.J.Wood & A.Lamb	✓									
Renanthera bella J.J.Wood	✓								✓	
Calanthe speciosa (Blume) Lindl.										✓
Calanthe otuhanica C.L.Chan & T.J.Barkman	✓									
Platanthera kinabaluensis Kraenzl. Ex Rolfe	✓		✓							
Thrixspermum kocyanii J.J.Wood & A.Lamb	✓									
Hetaeria hylophiloides (Carr) Ormerod & J.J.Wood	✓									
Liparis viridiflora (Blume) Lindl.					✓					
Liparis lacerata Ridl.	✓									
Liparis tricallosa Rchb.f.	✓		✓							
Malaxis punctata J.J.Wood			✓							
Malaxis subtiliscapa (J.J.Sm.) J.J.Wood			✓							
Malaxis graciliscapa Ames & C.Schweinf.			✓							
Malaxis ophrydis (J.König) Ormerod							✓			
Nervilia plicata (Andrews) Schltr.						✓				
Nervilia borneensis (J.J.Sm.) Schltr.						✓				
Kuhlhasseltia kinabaluensis Ames & C.Schweinf.			✓							
Kuhlhasseltia javanica J.J.Sm.			✓							
Oberonia patentifolia Ames & C.Schweinf.									✓	
Oberonia microphylla (Blume) Lindl.			✓							
Paphiopedilum dayanum (Robert Stone ex J.Dix) Stein			✓							
Paphiopedilum javanicum var. *virens* (Rchb.f.) Stein							✓			
Paphiopedilum hookerae var. *volonteanum* (Sander ex Rolfe) Braem	✓			✓						
Paphiopedilum sugiyamanum Cavestro						✓				
Pomatocalpa kunstleri (Hook.f.) J.J.Sm.						✓				
Peristylus gracilis Blume			✓							
Pholidota kinabaluensis Ames			✓							
Pholidota pectinata Ridl.			✓							
Pholidota clemensii Ridl.			✓				✓			
Pholidota sigmatochilus (Rolfe) J.J.Sm.	✓									
Phreatia densiflora Lindl.							✓			
Phreatia listrophora Ridl.			✓							
Plocoglottis lowii Rchb.f.		✓								

Table 2 (*continued*)

Species	Ultramafic Areas in Kinabalu Park								
	Mount Tambuyukon[3,6,7,10,11]	Panataran Valley[3,7,8,11]	Marai Parai[3,4,7,8,9,10,11,12,13,14,15,18]	Bukit Babi[3]	Bambangan[3,8,11]	Nalumad[7,8,10,11]	Mesilau[3,4,11,12,13,14]	Layang-Layang[3,11,14]	Hampuan Hill[3,4,7,11,13]	Lohan Valley[3,4,7,8,11,15]
Platanthera Stapf*ii* Kraenzl. Ex Rolfe	✓		✓							
Podochilus sciuroides Rchb.f.										✓
Podochilus tenuis Lindl.							✓			
Podochilus microphyllus Lindl.			✓							
Pteroceras teres (Blume) Holttum										✓
Peristylus hallieri J.J.Sm.			✓							
Poaephyllum podochiloides (Schltr.) Ridl.										✓
Robiquetia merrilliana (Ames) Lückel, M.Wolff & J.J.Wood			✓							
Spiranthes sinensis (Pers.) Ames			✓							
Schoenorchis buddleiflora (Schltr. & J.J.Sm.) J.J.Sm.			✓							
Schoenorchis paniculata Blume									✓	✓
Stichorkis lobongensis (Ames) J.J.Wood					✓				✓	
Stichorkis mucronata (Blume) J.J.Wood									✓	
Stichorkis pandurata (Ames) J.J.Wood					✓		✓			
Spathoglottis affinis de Vriese										✓
Spathoglottis gracilis Rolfe ex Hook.f.	✓		✓		✓					✓
Spathoglottis microchilina Kraenzl.			✓							
Spathoglottis kimballiana Hook.f.			✓							
Spathoglottis kimballiana var. *kimballiana*		✓								
Taeniophyllum rubrum Ridl.									✓	
Thelasis carnosa Ames & C.Schweinf.							✓			
Tainia purpureifolia Carr	✓									
Tainia speciosa Blume	✓									
Thrixspermum pensile Schltr.									✓	
Thrixspermum kocyanii J.J.Wood & A.Lamb	✓									
Thrixspermum triangulare Ames & C.Schweinf.	✓		✓							
Trichotosia brevipedunculata Ames & C.Schweinf.			✓				✓			
*Trichotosia aurea (*Ridl.*)* Carr	✓		✓							
Trichotosia ferox Blume			✓							
Trichoglottis smithii Carr									✓	✓
Trichoglottis maculata (J.J.Sm.) J.J.Sm.									✓	
Tropidia pedunculata Blume									✓	
Vanilla kinabaluensis Carr						✓				
Trichotosia microphylla Blume			✓							

Table 2 (*continued*)

Species	Ultramafic Areas in Kinabalu Park									
	Mount Tambuyukon[3,6,7,10,11]	Panataran Valley[3,7,8,11]	Marai Parai[3,4,7,8,9,10,11,12,13,14,15,18]	Bukit Babi[3]	Bambangan[3,8,11]	Nalumad[7,8,10,11]	Mesilau[3,4,11,12,13,14]	Layang-Layang[3,11,14]	Hampuan Hill[3,4,7,11,13]	Lohan Valley[3,4,7,8,11,15]
Vanilla pilifera Holttum								✓		
Vanilla havilandii Rolfe						✓				
Zeuxine gracilis (Breda) Blume										✓
Stigmatodactylus maraiparaiensis S.F.Md-Isa & A.S.Rob.			✓							
340	74	8	136	4	23	23	77	9	49	62

Sources: (Naturalis Bioportal, 2024)[3]; (Wood & Cribb, 1994)[4]; (Wood & van der Ent, 2012)[6]; (Wood et al., 2011a)[7]; (GBIF.org, 2017)[8]; (Md.-Isa et al., 2024)[9]; (van der Ent et al., 2014)[10]; (Wood et al., 2011b)[11]; (Natural History Museum, 2025)[12]; (Harvard University Herbaria & Libraries, 2025)[13]; (Royal Botanic Gardens, Kew, 2025)[14]; (JACQ - Virtual Herbaria, 2020)[15]; (Md.-Isa et al., 2025)[18]

Differences in species richness among ultramafic sites in Kinabalu Park may reflect unequal survey efforts rather than true biological variation. According to Table 2, Marai Parai hosts the highest orchid diversity among these sites, with 136 species recorded. A new species, *Stigmatodactylus maraiparaiensis* S.F.Md-Isa & A.S.Rob., has been formally described from Marai Parai, highlighting the site's importance for ongoing taxonomic discoveries and orchid diversity (Md.-Isa et al., 2025). Marai Parai is a protected area where minimal disturbance and intensive botanical surveys likely contribute to the high number of recorded species.

Orchid richness at other ultramafic sites is as follows: Mesilau (77), Mount Tambuyukon (74), Lohan Valley (62), Hampuan Hill (49), Bambangan (23), Nalumad (23), Layang-layang (9), Panataran Valley (8), and Bukit Babi (4). The low diversity documented at Bukit Babi likely reflects limited botanical survey efforts relative to other areas. However, *Coelogyne radioferens* Ames & C.Schweinf., is remarkably effective at Bukit Babi, where numerous orchids cannot perform well, and where it seems especially successful because instead of bending upright like typical inflorescent orchids, it grows in an arch, with its flowers well-spaced and having a remarkable lip, all features that increase the appeal of pollinating insects.

There are also species which are broadly adapted in several ultramafic locations. An example is *Bulbophyllum disjunctum* Ames & C.Schweinf., which has been reported in Mount Tambuyukon, Marai Parai and Mesilau. Other horticulturally viable plants are *Coelogyne hirtella* J.J.Sm. (Marai Parai, Bambangan, Mesilau), *Dilochia cantleyi* (Hook.f.) Ridl. (Mount Tambuyukon, Marai Parai, Mesilau), and *Epigeneium kinabaluense* (Ridl.) Summerh. (Mount Tambuyukon, Marai Parai, Bukit Babi).

Table 3
Summary of orchid diversity, endemism, and threats in ultramafic areas of Kinabalu Park

Site (Ultramafic Area)	Total Orchid Species	Endemic Species (Sabah/Kinabalu)	Non-endemic Species	% Endemic	Notes/ Threats
Mount Tambuyukon	74	22	52	29.7%	Human activities (trekking or research expeditions)
Panataran Valley	8	0	8	0	Illegal collection and climate change
Marai Parai	136	41	94	30.4%	Construction of a new route to Mount Kinabalu, unregulated hiking activities, and illegal poaching and collection
Bukit Babi	4	1	3	25%	Poaching and illegal collection for trade, human activities (hiking), climate change
Bambangan	23	1	22	4.3%	Limited monitoring, illegal collection, climate change
Nalumad	23	5	18	21.7%	Illegal harvesting and poaching for trade, and climate change
Mesilau	77	19	58	24.7%	Illegal harvesting and poaching for trade, and climate change
Layang-Layang	9	4	5	44.4%	Limited monitoring, illegal harvesting and poaching for trade, and climate change
Hampuan Hill	49	3	46	6.1%	Forest fires, illegal hunting for wild orchids, and agricultural activities
Lohan Valley	62	5	57	8.1%	Illegal harvesting and poaching for trade, and climate change

Despite the lack of an international assessment of the threat to extinction of most of these species, the existence of the endangered *Paphiopedilum dayanum* (Robert Stone ex J.Dix) Stein indicates that these habitats are conservation-urgent (Md.-Isa et al., 2024). To balance the ecosystem and ensure biodiversity conservation, there is a need to conserve the under-researched and species-poor sites like Panataran Valley, Bukit Babi, Bambangan, and Nalumad.

When contrasting the Mount Kinabalu and ultramafic sections of the Kinabalu Park, some interesting trends are visible. Mount Kinabalu alone sustains 564 species of orchids, including endemic taxa such as *Paphiopedilum rothschildianum* (Rchb.f.) Stein, while Kinabalu Park's ultramafic areas host 340 recorded species, highlighting their complementary roles in orchid conservation. The orchids in the two locations also have a different microhabitat: epiphytic orchids such as *Bulbophyllum salaccense* Rchb.f.,

Schoenorchis juncifolia Reinw. ex-Blume and *Pholidota carnea* (Blume) Lindl. need to have a particular host tree, such as *Schima wallichii* and *Lyonia ovalifolia* (Ai et al., 2023; Fardhani et al., 2019), whereas terrestrial orchids, such as *Apostasia wallichii* R.Br. and *Paphiopedilum dayanum* (Robert Stone ex J.Dix) Stein, depend on shaded, moist forests floors (van der Ent & Wood, 2013). The large gradient of elevation of Mount Kinabalu creates diverse microhabitats, which are home to orchid communities, which are not similar to those in the ultramafic soils of the park.

Mount Kinabalu has been extensively researched, and many botanical surveys have been carried out by research institutions. Conversely, the ultramafic regions in Kinabalu Park have a lesser amount of records on orchid-specific investigations, possibly because of the focus on the ecological observation and protection by the status of the UNESCO World Heritage site. Table 3 presents the total number of orchid species, their endemism status, and associated threats across different ultramafic habitats to facilitate comparison. As a result of unequal past sampling and inadequate data, the available values on species richness and endemism patterns of the species should not be taken as final ones. The categories of threats are based on published literature in the conservation and regional reports, as opposed to site-specific assessment.

THREATS TO ORCHIDS IN KINABALU PARK

Over time, concerns about the decline of orchid populations across Malaysia have become increasing (Sudin & Md.-Isa, 2024). Kinabalu Park harbours numerous endemic orchid species within its unique montane and ultramafic habitats. Orchids are also among the most endangered due to their complex life cycles, despite being one of the most diverse plant families. Their dependence on mycorrhizal fungi, specialised pollinators, and host trees makes conservation particularly challenging (Fay, 2018). Orchids are highly vulnerable to pressures such as over-collection, illegal trade, poaching, and climate change (Fay, 2018; Wraith et al., 2020). These threats could drive rare and endemic species to extinction if left unchecked, further disrupting the ecological balance of Kinabalu Park.

Over-collection

Over-collection has become a significant menace to the orchids in the Kinabalu Park. Orchids tend to over-harvest wild (orchid enthusiasts, commercial collectors and even researchers regularly over-harvest them) (Hului, 2015), controlled by market pressures, pleasing scents, morphological variation and horticultural interest (Go et al, 2020). The collection of orchids in sealed gardens or in green-houses are especially sought after by collectors, because they are looking to discover species that are inimitable or have particular forms and structures. Nevertheless, its slow rates of growth and ecologically extreme specialisation can lead to massive catastrophic losses of orchid populations since small-scale, yet frequent gathering can be conducted. Various species are sold at exorbitant prices in the local night markets and at the roadsides because of their charisma.

Common genera are *Dendrobium*, *Coelogyne*, *Paphiopedilum* (lady's slipper orchids), *Gramatophyllum* (tiger orchids), *Phalaenopsis*, and some species of *Vanda* (Chadburn & Saputra, 2021; Chacko, 2025). Illegal harvesting still poses a threat to the endangered species regardless of the current laws, and it affects not only the genetic diversity but also the wildness population.

Illegal Trade and Poaching

Another great threat is the illegal trade. Endemic, rare orchids- especially *Paphiopedilum* and *Phalaenopsis* species are very expensive in the international market and fetch high prices. In the 2024 IUCN Red List assessment, 99% out of the 85 species of *Paphiopedilum* assessed are threatened with extinction and overexploitation and the horticultural trade was found to be the main factor (IUCN, 2024). One such example is *Paphiopedilum rothschildianum,* which has been reported to be being illegally collected in the wild by commercial traders (CITES, 2026; Rankou, 2015). All orchids are listed under CITES, and over 70% of CITES-listed plant species are orchids (Fay, 2018). Most Orchidaceae are classified under Appendix II, which allows international trade under permits and Non-Detriment Findings (NDFs), while some genera, such as *Paphiopedilum,* are in Appendix I, prohibiting commercial trade entirely. Nonetheless, orchids continue to be harvested and traded internationally for ornamental, medicinal, and culinary purposes, often without the necessary permits. Market surveys in Southeast Asia document cross-border trade of hundreds of wild-collected orchid species, particularly high-value *Paphiopedilum,* with illegal sales increasingly facilitated through e-commerce and social-media platforms despite regulatory (Phelps & Webb, 2015).

Even with legal protections, orchids are poached for international markets, including Taiwan, China, and Europe (Fay, 2015). Local, national, and international markets collectively trade thousands of orchid species, both legally and illegally, with impacts ranging from sustainable to destructive (Hinsley et al., 2018). Due to the absence of their fungal symbionts, most illegally harvested orchids fail to survive in cultivation (Makwela, 2021). Enforcement has become more difficult due to the rapid growth of online business platforms, which facilitate illegal sales. However, systematic monitoring studies of the online orchid trade in Sabah still remain limited.

Climate Change

Many orchid species, particularly those restricted to small elevational ranges, are directly threatened by climate change (Liu et al, 2024; Md.-Isa et al, 2018). These species are threatened by habitat loss, changes in rainfall patterns, desiccation, and disruption of temperature-humidity regimes, even in protected areas. High-elevation orchids in Kinabalu Park depend on cool, humid conditions, and rising temperatures are forcing species upslope, gradually reducing their available habitat. Helmet orchids (*Corybas* species), which inhabit

mossy high-mountain zones, are especially vulnerable due to their dependence on specific microclimatic conditions. Species that are unable to adapt or move higher up the slopes are at risk of local or total extinction.

Climate change also impacts the fungal community in the Borneo mountain system, which indirectly affects orchids by altering the distribution of their mycorrhizal partners (Geml et al., 2017; Kolanowska, 2023). This symbiosis is critical, particularly for endemic orchid species such as *Paphiopedilum rothschildianum*, which only occur exclusively on metal-rich soils at high elevations in Kinabalu Park (van der Ent et al., 2015c). As climate-induced changes reduce fungal growth or alter soil conditions, the recovery of orchids in their natural habitats will become increasingly difficult. In addition, climate change is exacerbating existing threats by reducing the availability of suitable habitat and increasing susceptibility to drought, fire, and invasive species.

STRATEGIES FOR SUSTAINABLE ORCHID CONSERVATION

Kinabalu Park has quite a vulnerable ultramafic flora, as it is highly endemic and specialised. The agencies of the government, such as Sabah Parks, Sabah Forestry Department (SFD), and Sabah Biodiversity Centre (SABC), are tasked with monitoring and protecting the wild orchids, but a lot of focus is paid to the regulation of the trade. They have worked towards the prevention of unlawful and unsustainable harvesting and controlling the legal trade of endemic orchids, including *Paphiopedilum*. Although conservation assessments use a variety of approaches to assess the risk of extinction, tools like Maximum Entropy (MaxEnt) modelling and IUCN Red List criteria are commonly used by researchers. The application of MaxEnt, environmental variable-based species distribution methods that utilise presence-only data have been used to conserve orchids in Sabah. As an example, Juiling et al. (2020) applied the MaxEnt to simulate potential distributions of 47 endemic orchid species to produce a species richness heatmap to support conservation priorities.

Associating orchid preservation with the UNESCO Global Geopark initiative may equally raise awareness of the ultramafic terrain of the Kinabalu Park internationally, and also other sustainable and responsible tourism. The main conservation priorities involve preventing over-collection, reducing human disturbance, and trying to control effects caused by climate change. A proper strategy involves both *in-situ* and *ex-situ* control, education of the communities and firm enforcement of the law with a view to protecting and restoring the wild populations, restoration of the habitats as well as engaging the local communities and policymakers.

In-situ and *Ex-situ* Conservation Strategies

Multiple conservation strategies are needed in the conservation of orchids, both *in-situ* (within natural ecosystems like parks and reserves) and *ex-situ* environments (within orchidaria, botanical gardens, seed banks, nurseries, and laboratories).

In-situ conservation focusses on conservation of the species in their natural environment, the ecological interactions between plants, animals, and the environment. The diversity of the orchids is the highest in Kinabalu Park (one of nine national parks in Sabah), with about ~711 reported species. Measures on conservation involve biodiversity vigilance, ecosystem preservation and limiting the possible adverse activities.

Simple and standardised survey designs that can be applied in remote ultramafic valleys should be used in long-term monitoring. This will include the drawing of permanent plots or transects at representative locations, which will be surveyed at intervals to monitor the presence of species, their state of habitat, and abundance. These methods draw a balance between scientific rigour and the logistical challenges of these difficult environments (Swarts & Dixon, 2017).

Threatened species need *ex-situ* plans. To conserve the germplasm, some of the techniques are tissue culture, micropropagation, and seed banking. Interest in plant success is gauged by survival and reproductive output as well as the retention of genetically representative material to the species. Research organisations including Universiti Malaysia Sabah, in collaboration with the Poring Orchid Conservation Centre in Kinabalu Park, propagate rare orchids to reintroduce them. As an example, an in vitro regeneration process of the Sabah endemic jewel orchid *Macodes limii* J.J.Wood & A.Lamb generated acclimatised plantlets that are later reintroduced to the Centre, demonstrating a successful reproduction and nursery handling (David et al., 2022).

Nevertheless, reintroductions pose a threat of genetic hazards such as outbreeding depression and the disappearance of local lineages. In an attempt to reduce this risk, the reintroduction programs employed local species that were specifically adapted to the target habitat and guaranteed genetic integrity, as well as reduced any potential adverse effects.

Education and Awareness through Communication

Community participation and education are important to the conservation of orchids. There is a cluster of local communities and organisations that give their contribution in the shape of labour, funding, and advocacy. Ignorance is a stimulating factor of black trade and excessive harvesting. Outreach programme–signage, ecotourism activities, as well as school learning–assists in minimising negative practices. Further opportunities to engage the public are offered by campaigns on social media, community science platforms, and documentaries. The conservation ambassadors work with the indigenous and local communities to establish sustainable sources of income, hence limiting the motivation to illegally harvest.

To illustrate, Sabah Parks has provided the Poring Hot Spring Orchid Adoption Programme, wherein the community is sensitised on the importance of protecting the endangered orchids and enabled them to participate in the conservation activities (Sabah Park, 2025). Increasing awareness through public campaigns can increase the long-term support of the forester community in the protection of orchids.

Strict Enforcement of Conservation Laws

It is necessary to implement stringent rules and police controls to protect orchids. Some of the challenges are the market demand and the inability to supervise the remote poaching locations. Enforcement of regulations governing the protected areas, under the Parks Enactment, state-level measures restricting the possession and transport of the protected plants, licensing access to the biological resources, and control of international trade by CITES are the policy instruments applicable to flatten the poaching of orchids in Sabah. These are measures which are in accordance with the requirements of the Convention on Biological Diversity, which advocates *in-situ* conservation and sustainable utilisation of wild species (Secretariat of the Convention on Biological Diversity, 2000).

Performing training on the identification and monitoring of the orchids and custom licenses of the forestry officers, park rangers, and other customs officials makes enforcement more effective. More financial resources are also required to enhance infrastructure and manpower. New technologies like online surveillance and reporting systems, as well as local communities, may reinforce the implementation of regulations and prevent crime.

The multi-agencies in Malaysia proves the efficacy of coordinated enforcement. In February 2025, law enforcement in Malaysia (General Operations Force) confiscated more than 4,000 orchids, valued at an estimated count of RM2 million (estimated at about $450,000 USD). A 59-year-old man involved in the trade was detained (FMT Reporters, 2025). In November 2024, a 57-year-old man was caught smuggling ornamental orchids on board with an approximate value of over RM1 million of the plants without proper permission in Kelantan; the suspect and the orchids were rounded up to be reviewed according to Section 5 of the Plant Quarantine Act 1976 (Act 167) (Malay Mail, 2024).

CONCLUSION

Kinabalu Park's ultramafic habitat is a highly specialised ecosystem, supporting a remarkably diverse and well-adapted flora, particularly orchids that exhibit physiological and ecological adaptation enabling them to thrive in metal-rich and nutrient-poor soils. Despite its ecological importance and high degree of endemism, which have attracted international attention, the habitat remains under severe anthropogenic stress, including over-collection, illegal trade, poaching, and the increasing impacts of climate change. There is therefore an urgent need to develop robust and coordinated conservation efforts to prevent further deterioration of these fragile environments. Priority measures include protecting habitats, strengthening both *in-situ* and *ex-situ* conservation management, enhancing public awareness and education, and reinforcing legal and institutional protection.

This paper highlights the ecological significance and richness of orchids within the ultramafic habitats in the Kinabalu Park; however, significant knowledge gaps remain.

Priority research areas include understanding orchid-mycorrhizal fungal interactions, developing climate-based models to predict changes in dispersal, and establishing long-term monitoring programmes to track populations and habitat dynamics. Bridging these gaps will strengthen the scientific foundation needed to design effective restoration strategies for one of the world's most unique orchid floras.

Further research is essential to enhance our understanding of the ecological processes and specific adaptations that characterise this ecosystem. Integrating such scientific insights into conservation management will strengthen the resilience of ultramafic habitats within Kinabalu Park and support the persistence of endemic orchid species. Ultimately, conserving these ecosystems is crucial for preserving biodiversity, maintaining ecological balance, and safeguarding Sabah's natural heritage for future generations.

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SUPPLEMENTARY DATA

Table S1
List of orchid species recorded in the ultramafic area of Sabah, Malaysia

Species	Ultramafic Areas in Sabah			
	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Aeridostachya macrophylla (Ames & C.Schweinf.) J.J.Wood	✓				
Ascidieria longifolia (Hook.f.) Seidenf.	✓				
Ascidieria grandis (Ridl.) J.J.Wood	✓				
Ascidieria cymbidiifolia (Ridl.) W.Suarez & Cootes	✓				
Arundina graminifolia (D.Don) Hochr.	✓				
Aphyllorchis montana Rchb.f.	✓				
Appendicula clemensiorum J.J.Wood	✓				
Appendicula calcarata Ridl.	✓				
Appendicula tubilabia J.J.Wood	✓				
Appendicula tembuyukenensis J.J.Wood	✓				
Acanthophippium lilacinum J.J.Wood & C.L.Chan	✓				
Arachnis calcarata Holttum	✓				
Bromheadia longifolia Kruiz. & de Vogel	✓				
Bulbophyllum lemniscatoides Rolfe	✓				
Bulbophyllum teres Carr	✓				
Bulbophyllum proculcastris J.J.Verm.	✓				
Bulbophyllum retrorsum J.J.Verm. & A.Lamb	✓				
Bulbophyllum microglossum Ridl.	✓				
Bulbophyllum chanii J.J.Verm. & A.Lamb	✓				
Bulbophyllum depressum King & Pantl.	✓				
Bulbophyllum longhutense J.J.Sm.	✓				
Bulbophyllum ionophyllum J.J.Verm.	✓				
Bulbophyllum calceolus J.J.Verm.	✓				
Bulbophyllum dearei W.Watson	✓				
Bulbophyllum pelicanopsis J.J.Verm. & A.Lamb	✓				
Bulbophyllum puntjakense J.J.Sm.	✓				
Bulbophyllum schefferi (Kuntze) Schltr.	✓				
Bulbophyllum undecifilum J.J.Sm.	✓				
Campanulorchis leiophylla (Lindl.) Y.P.Ng & P.J.Cribb	✓				
Campanulorchis pellipes (Rchb.f. ex Hook.f.) Y.P.Ng & P.J.Cribb	✓				

Table S1 (*continued*)

Species	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Ultramafic Areas in Sabah			
		Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Chroniochilus virescens (Ridl.) Holttum	✓				
Chroniochilus minimus (Blume) J.J.Sm.	✓				
Chamaeanthus brachystachys Schltr.	✓				
Calanthe lambii P.J.Cribb	✓				
Calanthe vestita Wall. ex Lindl.	✓				
Calanthe woodii P.J.Cribb	✓				
Calanthe musa-amanii J.J.Wood	✓				
Calanthe otuhanica C.L.Chan & T.J.Barkman	✓				
Collabium ovalifolium (Ames & C.Schweinf.) J.J.Wood	✓				
Collabium simplex Rchb.f.	✓				
Corybas piliferus J.Dransf.	✓				
Cleisocentron abasii Cavestro	✓				
Cystorchis aphylla Ridl.	✓				
Coelogyne latiloba de Vogel	✓				
Coelogyne swaniana Rolfe	✓				
Coelogyne dayana Rchb.f.	✓				
Coelogyne pandurata Lindl.	✓				
Coelogyne septemcostata J.J.Sm.	✓				
Crepidium graciliscapum (Ames & C.Schweinf.) Szlach.	✓				
Cymbidium borneense J.J.Wood	✓				
Cymbidium tracyanum L.Castle	✓				
Cymbidium lowianum (Rchb.f.) Rchb.f.	✓				
Dilochia parviflora J.J.Sm.	✓				
Dendrobium serena-alexianum J.J.Wood & A.Lamb	✓				
Dendrobium cymboglossum J.J.Wood & A.Lamb	✓				
Dendrobium hirsutifolium J.J.Wood	✓				
Dendrobium beamanianum J.J.Wood & A.Lamb	✓				
Dendrobium kentrophyllum Hook.f.	✓				
Dendrobium kiauense Ames & C.Schweinf.	✓				
Dendrobium indivisum var. *pallidum* Seidenf.	✓				
Dendrochilum pseudoscriptum T.J.Barkman & J.J.Wood	✓				
Dendrochilum cruciforme var. *longicuspum* J.J.Wood	✓				
Dendrobium maraiparense J.J.Wood & C.L.Chan	✓				
Dendrobium lohanense J.J.Wood	✓				

Table S1 (*continued*)

Species	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Ultramafic Areas in Sabah			
		Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Dendrobium grande Hook.f.	✓				
Dendrobium linguella Rchb.f.	✓				
Dendrobium jiewhoei J.J.Wood & C.L.Chan	✓				
Dendrobium sanguinolentum Lindl.	✓				
Dendrobium cymbulipes J.J.Sm.	✓				
Grammatophyllum speciosum Blume	✓				
Habenaria koordersii J.J.Sm.	✓				
Habenaria setifolia Carr	✓				
Hetaeria hylophiloides (Carr) Ormerod & J.J.Wood	✓				
Hetaeria grandiflora Ridl.	✓				
Hetaeria rhombipetala Ormerod & J.J.Wood	✓				
Hetaeria elata Hook.f.	✓				
Hylophila lanceolata (Blume) Miq.	✓				
Jejewoodia jiewhoei (J.J.Wood & Shim) Szlach.	✓				
Lepidogyne longifolia (Blume) Blume	✓				
Liparis acaulis Schltr.	✓				
Lecanorchis multiflora J.J.Sm.	✓				
Nervilia borneensis (J.J.Sm.) Schltr.	✓				
Odontochilus hydrocephalus (J.J.Sm.) J.J.Wood	✓				
Gastrodia spatulata (Carr) J.J.Wood	✓				
Gastrodia javanica (Blume) Lindl.	✓				
Galeola nudifolia Lour.	✓				
Epigeneium treacherianum (Rchb.f. ex Hook.f.) Summerh.				✓	
Epigeneium treacherianum (Rchb.f. ex Hook.f.) Summerh.				✓	
Epipogium roseum (D.Don) Lindl.	✓				
Eulophia zollingeri (Rchb.f.) J.J.Sm.	✓				
Eulophia graminea Lindl.	✓				
Eria floribunda Lindl.	✓				
Eria augustifolia Ridl.	✓				
Eria borneensis Rolfe	✓				
Malaxis ophrydis (J. König) Ormerod	✓	✓			
Malaxis crepidium Bakh.f	✓				
Malaxis damusica (J.J.Sm.) Ames			✓		
Malaxis commelinifolia (Zoll. & Moritzi) Kuntze			✓		

Table S1 (*continued*)

Species	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Ultramafic Areas in Sabah			
		Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Chrysoglossum viridiflora Hook.f.	✓				
Chrysoglossum reticulatum Carr	✓				
Ceratostylis subulata Blume	✓				
Ceratostylis radiata J.J.Sm.	✓				
Liparis aurantiorbiculata J.J.Wood & A.Lamb	✓				
Stereosandra javanica Blume	✓				
Porrorhachis galbina (J.J.Sm.) Garay	✓				
Pholidota sigmatochilus J.J.Sm.	✓				
Pennilabium kidmancoxii J.J.Wood	✓				
Paphiopedilum kolopakingii Fowlie	✓				
Paphiopedilum sugiyamanum Cavestro	✓				
Paphiopedilum lowii (Lindl.) Stein	✓				
Peristylus candidus J.J.Sm.	✓				
Pholidota carnea (Blume) Lindl.	✓				
Pinalia latiuscula (Ames & C.Schweinf.) J.J.Wood	✓				
Phaius tankervilleae (Banks) Blume	✓				
Phaius reflexipetalus J.J.Wood & Shim	✓				
Podochilus serpyllifolius (Blume) Lindl.	✓				
Stichorkis lobongensis (Ames) J.J.Wood	✓				
Stichorkis pandurata (Ames) J.J.Wood	✓				
Spathoglottis microchilina Kraenzl.	✓				
Trichotosia aurea (Ridl.) Carr	✓				
Trichotosia brevipedunculata (Ames & C.Schweinf.) J.J.Wood	✓				
Tainia malayana J.J.Sm.	✓				
Tainia scapigera (Hook.f.) J.J.Sm.	✓				
Thrixspermum acuminatissimum (Blume) Rchb.f.	✓				
Tropidia saprophytica J.J.Sm.	✓				
Trichoglottis sitihasmahae J.J.Wood & A.Lamb	✓				
Trichoglottis tenuis Ames & C.Schweinf.	✓				
Dendrolirium ornatum Blume	✓				
Dendrobium spectatissimum Rchb.f.	✓				
Dendrobium sinsuronense J.J.Wood	✓				
Dendrobium alabense J.J.Wood	✓				
Dendrobium singulare Ames & C.Schweinf.	✓				

Table S1 (*continued*)

Species	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Ultramafic Areas in Sabah			
		Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Dendrobium oblongum Ames & C.Schweinf.	✓				
Dendrobium tawaiense J.J.Wood			✓		
Didymoplexiella kinabaluensis (Carr) Seidenf.	✓				
Ania borneensis (Rolfe) Senghas	✓				
Vanda helvola Blume	✓				
Macropodanthus sabahensis J.J.Wood & A.Lamb					
Mycaranthes magnicallosa (Ames & C.Schweinf.) J.J.Wood	✓				
Mycaranthes major (Stapf) J.J.Wood	✓				
Neuwiedia borneensis de Vogel	✓				
Oeceoclades pulchra (Thouars) P.J.Cribb & M.A.Clem.	✓				
Paphiopedilum rothschildianum (Rchb.f.) Stein	✓				
Phaius pauciflorus subsp. sabahensis J.J.Wood & A.Lamb	✓				
Porpax borneensis J.J.Wood & A.Lamb	✓	✓	✓		
Platanthera saprophytica J.J.Sm.	✓				
Renanthera bella J.J.Wood	✓				
*Crepidium metallicum (*Rchb.f.*)* Szlach.	✓		✓		
Dendrobium hamaticalcar J.J.Wood & Dauncey	✓				
Trichotosia mollicaulis (Ames & C.Schweinf.) J.J.Wood	✓				
Vanilla kinabaluensis Carr	✓				
Apostasia nuda R.Br.	✓		✓		
Apostasia odorata Blume	✓				
Coelogyne cuprea var. planiscapa J.J.Wood & C.L.Chan	✓				
Coelogyne monilirachis Carr	✓				
Coelogyne moultonii J.J.Sm.	✓				
Coelogyne exalata Ridl.	✓				
Coelogyne incrassata var. *valida* J.J.Sm.			✓		
Coelogyne verrucosa S.E.C.Sierra			✓		
Phalaenopsis maculata Rchb.f.			✓		
Paraphalaenopsis labukensis Shim, A.Lamb & C.L.Chan	✓	✓			
Platanthera crassinervia (Ames & C.Schweinf.) J.J.Sm.	✓				
Platanthera stapfii Kraenzl. ex Rolfe	✓				
Platanthera kinabaluensis Kraenzl. ex Rolfe	✓				
Dendrochilum planiscapum Carr	✓				
Dendrochilum angustilobum Carr	✓				

Table S1 (*continued*)

Species	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Ultramafic Areas in Sabah			
		Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Dendrochilum stachyodes (Ridl.) J.J.Sm.	✓				
Thecopus secunda (Ridl.) Seidenf.			✓		
Bulbophyllum placochilum J.J.Verm.	✓				
Bulbophyllum tenompokense J.J.Sm.	✓				
Bulbophyllum lambii J.J.Verm.	✓		✓		
Microssacus longicalcaratus Ames & C.Schweinf.	✓				
Bulbophyllum vinaceum Ames & C.Schweinf.	✓				
Bulbophyllum scabrum J.J.Verm. & A.Lamb	✓				
Bulbophyllum sigmoideum Ames & C.Schweinf.	✓				
Bulbophyllum lordoglossum J.J.Verm. & A.Lamb	✓				
Bulbophyllum dulitense Carr	✓				
Bulbophyllum koyanense Carr	✓				
Bulbophyllum gibbsae Rolfe	✓				
Bulbophyllum crassicaydatum Ames & C.Schweinf.	✓				
Bulbophyllum longimucronatum Ames & C.Schweinf.	✓				
Bulbophyllum minutiflorum Ames & C.Schweinf.	✓				
Bulbophyllum botryophorum Ridl.			✓		
Bulbophyllum flammuliferum Ridl.		✓			
Bulbophyllum limbatum Lindl.			✓		
Bulbophyllum sopoetanense Schltr.	✓				
Bulbophyllum lanceolatum Ames & C.Schweinf.	✓				
Bulbophyllum macrochilum Rolfe		✓			
Bulbophyllum deltoideum Ames & C.Schweinf.	✓				
Bulbophyllum reflexum Ames & C.Schweinf.	✓				
Bulbophyllum acuminatum (Ridl.) Ridl.			✓		
Bulbophyllum fascinator (Rolfe) Rolfe	✓				
Peristylus hallieri J.J.Sm.	✓				
Dendrobium pandaneti Ridl.	✓				
Corybas serpentinus J.Dransf.				✓	✓
Corybas crenulatus J.J.Sm.	✓				
Apostasia wallichii R.Br.		✓	✓	✓	✓
Liparis lingulata Ames & C.Schweinf.	✓				
Liparis anopheles J.J.Wood	✓				
Bulbophyllum flavescens (Blume) Lindl.	✓		✓		

Table S1 (*continued*)

Species	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Ultramafic Areas in Sabah			
		Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Tainia purpureifolia Carr	✓				
Schoenorchis micrantha Blume	✓				
Coelogyne rigidiformis Ames & C.Schweinf.	✓				
Coelogyne genuflexa Ames & C.Schweinf.	✓				
Coelogyne clemensii Ames & C.Schweinf.	✓				
Coelogyne clemensii var. *angustifolia* Carr	✓				
Coelogyne clemensii var. *longiscapa* Ames & C.Schweinf.	✓				
Coelogyne tenompokensis Carr	✓				
Coelogyne obtusifolia Carr	✓				
Coelogyne craticulaelabris Carr	✓				
Coelogyne compressicaulis Ames & C.Schweinf.	✓				
Liparis elegans Lindl.	✓		✓		
Corymborkis veratrifolia (Reinw.) Blume	✓				
Pholidota clemensii Ames	✓				
Dendrochilum grandiflorum J.J.Sm.	✓				
Appendicula fractiflexa J.J.Wood	✓				
Appendicula pseudofractiflexa J.J.Wood			✓		
Appendicula torta Blume	✓				
Bulbophyllum marudiense Carr			✓		
Bulbophyllum coniferum Ridl.	✓				
Lecanorchis multiflora J.J.Sm.	✓				
Dendrobium roseobellum J.J.Wood	✓				
Bulbophyllum antenniferum Rchb.f.	✓				
Dendrochilum gibbsiae Rolfe	✓				
Cyrtosia javanica Blume	✓				
Coelogyne planiscapa var. *grandis* Carr	✓				
Coelogyne planiscapa var. *plicatissima* Ames & C.Schweinf.	✓				
Liparis parviflora (Blume) Lindl.	✓				
Anoectochilus integrilabris Carr	✓				
Coelogyne hirtella J.J.Sm.	✓				
Bulbophyllum coriaceum Ridl. ex Stapf	✓				
Coelogyne longibulbosa Ames & C.Schweinf.	✓				
Oberonia kinabaluensis K.Y.Lang & D.S.Deng	✓				
Cymbidium angustifolium Ames & C.Schweinf.	✓				

Table S1 (*continued*)

Species	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Ultramafic Areas in Sabah			
		Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Cymbidium dayanum Rchb.f.	✓				
Cymbidium elongatum J.J.Wood, Du Puy & Shim	✓				
Dendrochilum sublobatum Carr	✓				
Cionisaccus procerus (Ker Gawl.) M.A.Clem. & D.L.Jones	✓				
Eria macrophylla Ames & C.Schweinf.					
Eria carnosissima Ames & C.Schweinf.	✓				
Eria magnicallosa Ames & C.Schweinf.	✓				
Eria villosissima Rolfe	✓				
Eria pseudoleiophylla J.J.Wood	✓				
Eria pseudocymbiformis var. *hirsuta* J.J.Wood	✓				
Dendrochilum scriptum Carr	✓				
Appendicula cornuta Blume			✓		
Appendicula congesta Ridl. ex Stapf	✓				
Thrixspermum centipeda Lour.	✓				
Dilochia cantleyi (Hook.f.) Ridl.	✓				
Appendicula anceps Blume	✓				
Peristylus goodyeroides (D.Don) Lindl.	✓				
Liparis viridiflora (Blume) Lindl.	✓				
Trichotosia microphylla Blume	✓				
Chelonistele sulphurea var. *sulphurea* (Blume) Pfitzer	✓				✓
Neoclemensia spathulata Carr	✓				
Nephelaphyllum flabellatum Ames & C.Schweinf.	✓				
Nephelaphyllum pulchrum Blume	✓				
Dendrochilum dewindtianum var. *dewindtianum* W.W.Sm.	✓				
Podochilus tenuis (Blume) Lindl.	✓				
Podochilus lucescens Blume	✓		✓		
Trichotosia aporina (Hook.f.) Kraenzl.	✓				
Eria grandis Ridl. ex Stapf	✓				
Appendicula foliosa Ames & C.Schweinf.	✓				
Thrixspermum tanuis Ames & C.Schweinf.					
Thrixspermum crescentiforme Ames & C.Schweinf.	✓		✓		
Pantlingia lamrii J.J.Wood & C.L.Chan	✓				
Paphiopedilum hookerae var. *volonteanum (*Sander ex Rolfe*)* Braem	✓	✓			

Table S1 (*continued*)

Species	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Ultramafic Areas in Sabah			
		Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Paphiopedilum dayanum (Rchb.f.) Pfitzer	✓				
Entomophobia kinabaluensis (Ames) de Vogel	✓				
Eria obliqua (Lindl.) Lindl.	✓				
*Bulbophyllum membranifolium sub*sp. *membranifolium*	✓				
Dendrobium microglaphys Rchb.f.			✓		
Dendrobium sculptum Rchb.f.	✓				
Coelogyne radioferens Ames & C.Schweinf.	✓				
Taeniophyllum culiciferum Ridl.	✓				
Eria major Ridl. ex Stapf	✓				
Dendrobium concinnum Miq.	✓				
Bulbophyllum lobbii Lindl.	✓				
Trichoglottis magnicallosa Ames & C.Schweinf.	✓				
Dendrochilum crassum Ridl.	✓				
Eria robusta (Blume) Lindl.	✓				
Dimorphorchis rossii Fowlie		✓			
Dimorphorchis lowii (Benth. & Hook.f.) Rolfe	✓				
Microsaccus longicalcaratus Ames & C.Schweinf.	✓				
Dendrobium crumenatum Sw.	✓				
Cymbidium chloranthum Lindl.	✓				
Dendrochilum hologyne Carr	✓				
Octarrhena condensata (Ridl.) Holttum	✓				
Thelasis macrobulbon Ridl.	✓				
Eulophia spectabilis (Dennst.) Suresh	✓				
Spathoglottis kimballiana Hook.f.		✓		✓	
Spathoglottis plicata Blume	✓				
Bulbophyllum comberi J.J.Verm.	✓				
Phaius subtrilobus Ames & C.Schweinf.	✓				
Phaius tankervilleae (Banks ex L'Hér.) Blume	✓				
Dendrobium tridentatum Ames & C.Schweinf.	✓				
Bulbophyllum cornutum (Lindl.) Rchb.f.	✓				
Kuhlhasseltia kinabaluensis Ames & C.Schweinf.	✓				
*Acriopsis liliiflora (*J. Koenig) Ormerod	✓				
Malaxis kinabaluensis (Rolfe) Ames & C.Schweinf.	✓				
Dendrochilum subintegrum Ames	✓				

Table S1 (*continued*)

Species	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Ultramafic Areas in Sabah			
		Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Dendrochilum fimbriatum Ames	✓				
Dendrochilum kamborangense Ames	✓				
Bulbophyllum anaclastum J.J.Verm.	✓				
Bulbophyllum lygeron J.J.Verm.	✓				
*Thrixspermum trichoglottis (*Hook.f.*)* Kuntze	✓				
Coelogyne cuprea H.Wendl. & Kraenzl.	✓				✓
Epigeneium tricallosum Ames & C.Schweinf.	✓				
Epigeneium longirepens Ames & C.Schweinf.	✓				
Epigeneium kinabaluense (Ridl.) Summerh.	✓				
Dendrochilum longirachis Ames	✓				
Coelogyne venusta Rolfe	✓				
Thrixspermum magnicallosa Ames & C.Schweinf.	✓				
Thrixspermum triangulare Ames & C.Schweinf.	✓				
Vrydagzynea grandis Ames & C.Schweinf.	✓				
Vrydagzynea bicostata Carr	✓				
Vrydagzynea argentistriata Carr	✓				
Vrydagzynea elata Schltr.	✓				
Grammatophyllum kinabaluense Ames & C.Schweinf.	✓				
Bulbophyllum mutabile (Blume) Lindl.	✓				
Thelasis variabilis Ames & C.Schweinf.	✓				
Dendrochilum alpinum Carr	✓				
Zeuxine papillosa Carr	✓				
Dendrochilum gracilipes Carr	✓				
Dendrochilum graminoides Carr	✓				
Vanilla kinabaluensis Carr					✓
Vanilla sumatrana J.J.Sm.	✓				
Dendrochilum haslamii Ames	✓				
Chelonistele lurida Pfitzer	✓				
Liparis kinabaluensis J.J.Wood					
Liparis kamborangensis Ames & C.Schweinf.	✓				
Robiquetia crockerensis Wood & Lamb	✓				
Bulbophyllum breviflorum Ridl.	✓				
Dendrobium beamanianum J.J.Wood & A.Lamb	✓				
Liparis pandurata Ames	✓				

Table S1 (*continued*)

Species	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Ultramafic Areas in Sabah			
		Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Eria monophylla Schltr.	✓				
Thrixspermum amplexicaule (Blume) Rchb.f.	✓				
Dendrochilum pterogyne Carr	✓				
*Nervilia punctata (*Blume*)* Makino	✓				
Bromheadia divaricata Ames & C.Schweinf.	✓				
Bromheadia borneensis J.J.Sm. var. *borneensis*			✓		
Bromheadia brevifolia Ridl.	✓				
Peristylus gracilis Blume	✓				
Bulbophyllum obtusum (Blume) Lindl.	✓				
Spathoglottis aurea Lindl.	✓				
Phreatia densiflora (Blume) Lindl.	✓				
Coelogyne rupicola Carr	✓				
Dendrochilum transversum Carr	✓				
Paphiopedilum javanicum Pfitzer	✓				
*Eria ornata (*Blume*)* Lindl.	✓				
Bulbophyllum eximium Ames & C.Schweinf.	✓				
Bulbophyllum pergracile Ames & C.Schweinf.	✓				
Bulbophyllum cuneifolium Ames & C.Schweinf.	✓				
Bulbophyllum caudatisepalum Ames & C.Schweinf.	✓				
Dendrobium orbiculare J.J.Sm.	✓				
Goodyera rostellata Ames & C.Schweinf.	✓				
Habenaria setifolia Carr	✓				
Habenaria damaiensis J.J.Sm.	✓				
Trichoglottis collenetteae J.J.Wood, C.L.Chan & A.Lamb	✓				
Macodes limii J.J.Wood & A.Lamb		✓			
*Macodes petola (*Blume*)* Lindl.	✓				
Dendrochilum conopseum Ridl. ex Stapf	✓				
Nabaluia angustifolia de Vogel	✓				
Appendicula recondita J.J.Sm.	✓				
Flickingeria dura (J.J.Sm.) A.D.Hawkes	✓				
Trichoglottis kinabaluensis Rolfe	✓				
Ceratostylis ampullacea Kraenzl.	✓				
Coelogyne naja J.J.Sm.	✓				
Erythrodes latifolia Blume	✓				

Table S1 (*continued*)

Species	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Ultramafic Areas in Sabah			
		Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Microsaccus longicakaratus Ames & C.Schweinf.	✓				
Appendicula linearifolia Ames & C.Schweinf.	✓				
Vanda gibbsiae Rolfe	✓				
Chelonistele sulphurea var. *crassifolia (*Blume*)* Pfitzer	✓				✓
Arachnis longisepala (J.J.Wood) Shim & A.Lamb	✓	✓			
Arachnis flosaeris var. *gracilis* (L.) Rchb.f.	✓				
Agrostophyllum globigerum Ames & C.Schweinf.	✓				
Agrostophyllum saccatum Ridl.			✓		
Agrostophyllum stipulatum (Griff.) Schltr.			✓		
Agrostophyllum glumaceum Hook.f.			✓		
Agrostophyllum bicuspidatum J.J.Sm.	✓				
Bulbophyllum dryas Ridl.	✓				
Thrixspermum pensile Schltr.	✓				
Chelonistele amplissima (Ames & C.Schweinf.) Carr	✓				
Dendrobium lamelluliferum J.J.Sm.	✓				
Anoectochilus monicae J.J.Wood	✓				
Goodyera rubicunda (Blume) Lindl.	✓				
Bulbophyllum hemiprionotum J.J.Verm. & A.Lamb	✓				
Bulbophyllum heldiorum J.J.Verm.	✓				
Oberonia triangularis Ames & C.Schweinf.	✓				
Oberonia patentifolia Ames & C.Schweinf.	✓				
Oberonia affinis Ames & C.Schweinf.	✓				
Bulbophyllum dracunculus J.J.Verm.	✓				
Flickingeria bicarinata (Ames & C.Schweinf.) A.D.Hawkes	✓				
Calanthe ovalifolia Ridl.	✓				
Calanthe tenuis Ames & C.Schweinf.	✓				
Calanthe cuneata Ames & C.Schweinf.	✓				
Calanthe kinabaluensis Rolfe.	✓				
Calanthe pulchra (Blume) Lindl.	✓				
Calanthe gibbsiae Rolfe	✓				
Bulbophyllum apodum Hook.f.	✓				
Nabaluia clemensii Ames	✓				
Dendrobium bifarium Lindl.	✓				
Appendicula pendula Blume	✓				

Table S1 (*continued*)

Species	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Ultramafic Areas in Sabah			
		Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Peristylus grandis Blume	✓				
Bulbophyllum alatum J.J.Verm.	✓				
Cleisostoma inflatum (Rolfe) Garay	✓				
Cleisostoma striatum (Rchb.f.) Garay	✓				
Taeniophyllum obtusum Blume	✓				
Spathoglottis gracilis Rolfe ex Hook.f.	✓	✓			
Coelogyne rochussenii de Vriese	✓				
Liparis grandis Ames & C.Schweinf.	✓				
Thrixspermum pardale (Ridl.) Schltr.	✓				
Neuwiedia zolingeri var. *javanica* (J.J.Sm.) de Vogel		✓			
Neuwiedia veratrifolia Blume	✓				
Phalaenopsis amabilis (L.) Blume	✓				
Bulbophyllum rhizomatosum Ames & C.Schweinf.	✓				
Bulbophyllum pugilanthum J.J.Wood	✓				
Liparis caespitosa (Thouars) Lindl.	✓				
Eria hyacinthoides (Blume) Lindl.	✓				
Phreatia monticola Schltr.	✓				
Eria leiophylla Lindl.	✓				
Eria nutans Lindl.	✓				
Eria kinabaluensis Rolfe	✓				
Coelogyne rhabdobulbon Schltr.	✓				
Bulbophyllum mandibulare Rchb.f.	✓				
Geodorum densiflorum (Lam.) Schltr.	✓				
Dendrobium maraiparense Wood & C.L.Chan	✓				
Bulbophyllum anguliferum Ames & C.Schweinf.	✓				
Bulbophyllum latisepalum Ames & C.Schweinf.	✓				
Trichotosia ferox Blume	✓				
Bulbophyllum purpurascens Teijsm. & Binn.	✓				
Dendrochilum lancilabium Ames	✓				
Aphyllorchis pallida Blume	✓				
Taeniophyllum stella Carr	✓				
Gastrodia grandilabris Carr	✓				
Bulbophyllum kestron J.J.Verm. & A.Lamb	✓				
Liparis lobongensis Ames	✓				

Table S1 (*continued*)

Species	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Ultramafic Areas in Sabah			
		Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Appendicula divaricata Ames & C.Schweinf.	✓				
Dendrobium kurashigei T. Yukawa	✓				
Dendrochilum alatum Ames	✓				
Ceratostylis longisegmenta Ames & C.Schweinf.					
Ceratostylis crassilingua Ames & C.Schweinf.	✓				
Pholidota gibbosa (Blume) de Vriese	✓				
Coelogyne kinabaluensis Ames & C.Schweinf.	✓				
Coelogyne radioferens Ames & C.Schweinf.	✓				
Taeniophyllum rubrum Ridl.	✓				
Thelasis carnosa Ames & C.Schweinf.	✓				
Robiquetia transversisaccata (Ames & C.Schweinf.) J.J.Wood	✓				
Dendrochilum pallideflavens Blume	✓				
Dendrobium patentilobum Ames & C.Schweinf.	✓				
Dendrochilum tenompokense Carr	✓				
Oberonia rubra Ridl.	✓				
Coelogyne subintegra J.J.Sm.					✓
Tainia paucifolia (Breda) J.J.Sm.	✓				
*Dilochia rigida (*Ridl.*)* J.J.Wood	✓				
Schoenorchis juncifolia Reinw.	✓				
Coelogyne tenompokensis Carr	✓				
Cryptostylis clemensii J.J.Sm.	✓				
Didymoplexis kinabaluensis Carr	✓				
Phreatia plantaginifolia (K.D.Koenig) Ormerod	✓				
Dendrobium villosulum Lindl.	✓				
Peristylus ciliatus Carr	✓				
Appendicula maggnibracteata Ames & C.Schweinf.	✓				
Appendicula longirostrata Ames & C.Schweinf.	✓				
Flickingeria fimbriata (Blume) A.D.Hawkes	✓				
Corybas kinabaluensis Carr	✓				
Eria saccifera Hook.f.	✓		✓		
Bulbophyllum catenarium Ridl.	✓				
Bulbophyllum apheles J.J.Verm.	✓				
Spiranthes sinensis (Pers.) Ames	✓				
Flickingeria pseudoconvexa (Ames) A.D.Hawkes	✓				

Table S1 (*continued*)

Species	Ultramafic Areas in Sabah			
	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Goodyera ustulata Carr	✓				
Cleisocentron merrillianum (Ames) Christenson	✓				
Dendrochilum joclemensii Ames	✓				
Coelogyne papillosa Ridl.	✓				
Thelasis pygmaea (Griff.) Blume	✓				
Nephelaphyllum verruculosum Carr	✓				
Coelogyne pulverula Teijsm. & Binn.	✓				
Phreatia sulcata (Blume) J.J.Sm.	✓				
Dendrochilum acuiferum Carr	✓				
Bulbophyllum montense Ridl. ex Stapf	✓				
Bromheadia finlaysoniana (Lindl.) Miq.	✓		✓		
Bulbophyllum lissoglossum J.J.Verm.	✓				
Thecostele alata (Roxb.) C.S.P.Parish & Rchb.f.	✓				
Bulbophyllum scheffleri Schltr.	✓				
Myrmechis kinabaluensis Carr	✓				
Dendrochilum dewindtianum W.W.Sm.	✓				
Dendrochilum pallideflavens var. *pallidiflavens* Blume	✓				
Calanthe speciosa (Blume) Lindl.	✓				
Dendrochilum lacteum Carr	✓				
Bulbophyllum membranaceum Teijsm. & Binn.	✓				
Cryptostylis arachnites (Blume) Hassk.	✓				
Chelonistele kinabaluensis (Rolfe) de Vogel	✓				
Octarrhena parvula Thwaites	✓				
Coelogyne plicatissima Ames & C.Schweinf.	✓		✓		
Bromheadia truncata Seidenf.	✓				
Bulbophyllum ceratostylis J.J.Sm.	✓				
Liparis viridicallus Holttum	✓				
Dendrobium ventripes Carr	✓				
Bulbophyllum salaccense Rchb.f.	✓				
Eria pseudocymbiformis var. *hirsuta* J.J.Wood	✓				
Aerides odorata Lour.	✓				
Pholidota carnea var. *carnea* (Blume) Lindl.	✓				
Appendicula minutiflora Ames & C.Schweinf.	✓				
Pteroceras spathibrachiatum (J.J.Sm.) Garay	✓				

Table S1 (*continued*)

Species	Ultramafic Areas in Sabah			
	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Eria cymbidifolia Ridl.	✓				
Bulbophyllum disjunctum Ames & C.Schweinf.	✓				
Oberonia equitans (Thouars) Schltr.	✓				
Mischobulbum scapigerum (Hook.f.) Schltr.	✓				
Dendrochilum imbricatum Ames	✓				
Eria farinosa K.Y.Lang & D.S.Deng	✓				
Pholidota pectinata Ames	✓				
Peristylus brevicalcar Carr	✓				
Zeuxine strateumatica (L.) Schltr.	✓				
Dendrochilum angustipetalum Ames	✓				
Taeniophyllum esetiferum J.J.Sm.	✓				
Calanthe triplicata (Willemet) Ames	✓				
Goodyera kinabaluensis Rolfe	✓				
Malleola kinabaluensis Ames & C.Schweinf.	✓				
Arundina graminifolia (D.Don) Hochr.	✓				
Liparis latifolia (Blume) Lindl.	✓				
Zeuxine benguetensis (Ames) Ames	✓				
Bulbophyllum hyalosemoides J.J.Verm. & P.O'Byrne	✓				
Bulbophyllum lobbii subsp. *borneoborneense*	✓				
Eria latiuscula Ames & C.Schweinf.	✓				
Bulbophyllum mahakamense J.J.Sm.	✓				
Bulbophyllum trifolium Ridl.	✓				
Liparis atrosanguinea Ridl.	✓				
Peristylus kinabaluensis Carr	✓				
Platanthera gibbsiae Rolfe	✓				
Platanthera crassinervia, (Ames & C.Schweinf.) J.J.Sm.	✓				
Platanthera borneensis (Ridl.) J.J.Wood	✓				
Platanthera angustata (Blume) Lindl.	✓				
Bromheadia ensifolia J.J.Sm.	✓				
Aphyllorchis borneensis Schltr.	✓				
Phaius baconii J.J.Wood & Shim	✓				
Appendicula kinabaluensis J.J.Sm.	✓				
Pteroceras biserratum (Ridl.) Holttum	✓				
Dendrobium minimum Ames & C.Schweinf.	✓				

Table S1 (*continued*)

Species	Mount Kinabalu[1,3,4,7,8,11,12,13,14,15,16,17]	Ultramafic Areas in Sabah			
		Meliau Range[2,7,11]	Mount Tawai[3,4]	Bidu-bidu Hills[2,5,8]	Mount Silam[2,4]
Bulbophyllum muscohaerens J.J.Verm. & A.Lamb	✓				
Dendrobium salaccense (Blume) Lindl.	✓				
Dendrobium singkawangense J.J.Sm.	✓				
Dendrochilum exasperatum Ames	✓				
Eria cepifolia Ridl.	✓				
Dendrobium cymbulipes J.J.Sm.	✓				
Acriopsis javanica Reinw. ex Blume var. *javanica*			✓		
Acriopsis indica Wight	✓				
Eria oblitterata (Blume) Rchb.f.	✓				
Malaxis punctata J.J.Wood	✓				
Malaxis subtiliscapa (J.J.Sm.) J.J.Wood	✓				
Dendrochilum corrugatum J.J.Sm.	✓				
Bulbophyllum pocillum J.J.Verm.	✓				
Liparis condylobulbon Rchb.f.	✓				
Cryptostylis acutata J.J.Sm.	✓				
Eria pseudocymbiformis J.J.Wood	✓				
Dendrochilum minimiflorum Carr	✓				
Epigeneium radicosum (Ridl.) Summerh.	✓				
Malaxis multiflora Ames & C.Schweinf.	✓				
Malaxis graciliscapa Ames & C.Schweinf.	✓				
Malaxis variabilis Ames & C.Schweinf.	✓				
Bulbophyllum minutulum Ridl. ex Burkill & Holttum	✓				
Eria jenseniana J.J.Sm.	✓				
Bulbophyllum nubinatum J.J.Verm.	✓				
563	527	13	31	5	7

Sources: (BRAHMS: Singapore Herbarium, 2024)[1]; (Chung, 2006)[2]; (Naturalis Bioportal, 2024)[3]; (Wood & Cribb, 1994)[4]; (van der Ent & Wood, 2013)[5]; (Wood et al., 2011a)[7]; (GBIF.org, 2017)[8]; (Wood et al., 2011b)[11]; (Natural History Museum, 2025)[12]; (Harvard University Herbaria & Libraries, 2025)[13]; (Royal Botanic Gardens, Kew, 2025)[14]; (JACQ - Virtual Herbaria, 2020)[15]; (Juiling et al., 2020)[16]; (Wood et al, 1993)[17]

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Review Article

A Review on the Optimisation of Enzymatic Treatment in Tropical Fruit Juice: Impacts on Physicochemical and Functional Properties

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ABSTRACT

The production of fruit juice often faces challenges in achieving high juice quality due to its high viscosity, pectin, and polysaccharide content. Enzymatic treatment has proved to be one of the most effective approaches for improving the juice yield, quality and nutritional properties. Enzymes such as pectinase, cellulase, and hemicellulase are commonly used in fruit juice production to degrade cell wall components, thereby enhancing the stability and quality of fruit juice. Optimisation of enzymatic treatment conditions, such as enzyme concentration, incubation time, and temperature, using a second-order central composite design (RSM), was crucial in achieving the desired quality outcome. From an extensive collection of literature and research, this comprehensive review explores the impact of optimised enzymatic treatment conditions on the physicochemical properties, including juice yield, viscosity, clarity, turbidity, total soluble solids (TSS), and the bioactive compounds of antioxidant activity and total phenolic content (TPC) of tropical fruit juices. Key findings suggest that different fruits all have different enzymatic treatment conditions due to the dependency of this treatment towards the pectin, cellulose, hemicellulose and other polysaccharide contents in the fruit. This selected treatment shows effectiveness in tackling the stated issues.

Keywords: Enzymatic treatment, juice quality, bioactive compounds, response surface methodology, tropical juice

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INTRODUCTION

Tropical fruits are excellent and nutritious natural resources that grow in warm and

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humid tropical climates, such as in Central America, South America, Southeast Asia and Middle Africa (López-Martínez et al., 2023; Sarkar et al., 2023; Sayago-Ayerdi et al., 2021). Tropical fruits come in a wide variety, ranging from the succulent sweetness of mangoes to the creamy richness of avocados with vibrant flavours, rich colours, and diverse textures (Paull & Duarte, 2025). Tropical fruit is an important key segment of global agriculture and trade, provided that it is widely consumed, supporting both local and international market demand (FAO, 2024; Mohamed et al., 2011).

There was a significant global increase in the production and consumption of tropical fruits nationwide. The surge is primarily attributed to the recognition of their nutritional and health benefits, growing demand for exotic flavours, and increased awareness of their health-promoting properties (Sayago-Ayerdi et al., 2021). The fact that tropical fruits are an abundant source of essential nutrients, offering numerous health benefits to the human body, also fuels the growing interest in these fruits among consumers. Overall, tropical fruits provide essential vitamins, including A, B, C, and E (Paull & Duarte, 2025), as well as minerals such as potassium, magnesium, and manganese (Paull & Duarte, 2025). It is also a good source of dietary fibre, which supports digestive health, lowers cholesterol levels, and promotes a healthy gut (Chuacharoen et al., 2021; Ioniță-Mîndrican et al., 2022). In addition, tropical fruits contain antioxidants, such as flavonoids and carotenoids, that help protect cells from damage (Sayago-Ayerdi et al., 2021), as well as phytonutrients with anti-inflammatory and immune-boosting properties (Sayago-Ayerdi et al., 2021).

According to studies conducted by Sayago-Ayerdi et al. (2021) and Maia et al. (2019), roughly 50% of tropical and subtropical fruit production is designated for fresh fruit consumption, while the other half is utilised as processed products such as wines, dried fruit snacks, syrups, marmalades, flours, and tropical fruit beverages, which are among the most popular offerings. Due to their high-water content and delicate nature, many tropical fruits are highly perishable and are thus often transformed into beverages for preservation. This fragility is influenced by various factors, including high respiration rates, as tropical fruits tend to consume oxygen and emit carbon dioxide at a faster rate than other fruits. They are also susceptible to physical damage during harvesting, transportation, and storage (Bensi et al., 2020). These factors hasten their decay and vulnerability to microbial spoilage, as poor storing and handling of fruits make them easily contaminated with bacteria, fungi, and other microorganisms (Kusumaningrum et al., 2015; Zainalabidin et al., 2019).

ENZYMATIC TREATMENT OF FRUIT JUICE

Fruits contain complex polysaccharides such as pectin, starch and hydrophilic hydroxyl groups (-OH), which contribute to viscous juice, low juice yield, and inconsistent appearance when extracted using conventional methods (Toy et al., 2022). Although this method is widely used, it is often costly to maintain, energy-intensive, and prone to

cross-contamination (Vivek et al., 2019). Additionally, this method may produce turbid juice with suspended particles, which consumers consider low-quality juice. Toy et al. (2022) reported that a more clarified juice with a bright and transparent appearance is preferred as it is often associated with higher quality. To address these challenges, alternative methods such as cold pressing in pineapple and dragon fruit, hot extraction in banana, and enzymatic extraction in dragon fruit have been explored by several researchers (Dowerah et al., 2023; Khaksar et al., 2019; Ghorband & Joshi, 2023). In the literature, it was shown that enzymatic treatment using pectinases, cellulases, hemicellulases, amylases, and proteases (Michele, 2020) as tabulated in Table 1, is used to improve juice recovery and clarity at various stages of juice processing, including maceration, pressing, and clarification (García, 2018) as compared to cold and hot extraction (Joshi et al., 1991).

Table 1
Different types of enzymes and their function during enzymatic treatment

Enzyme	Subcategories	Functions	Ref
Pectinase	Pectin methylesterase (PME)	Methoxyl group (pectin) de-esterification	Garg et al. (2016)
	Protopectinase	Degradation of insoluble protopectin into soluble pectin	
	Polygalacturonase (PG)	Hydrolyses α-1,4-glycosidic linkages in polygalacturonic acid	
	Polymethylgalacturonase (PMG)	Hydrolyses α-1,4-glycosidic bonds	
Celllulase	β-glucosidase (BG)	Hydrolyses disaccharides and tetrasaccharides into glucose	Singh et al. (2016)
	Exo-β 1,4-cellobiohydrolases (CBH)	Hydrolyses polymers into simple sugars	
	Endo-β 1,4-glucanases (EG)	Breaking down non-covalent interactions (endocellulose)	
Hemicellulase	β-mannanase	Hydrolyses mannan-based hemicellulose	Brigham et al. (2018)
	β-mannosidase	Monno-oligomer hydrolyses into mannose	
	Xylanase	Hydrolyse the β-1,4 bond in xylan xylo-oligomer	
Protease		Hydrolysing peptide bonds into amino acids and polypeptide chains	Raveendran et al. (2018)
Tannase		Cleaving ester bonds in complex tannins to gallic acid	Méndez-Carmona et al. (2022)
Amylase	α-amylase	Breakdown of α-1,4-glycosidic bond (start) into sugars	Mojsov (2016)
	β-amylase	Catalysing the hydrolysis of the α-1, 4-glycosidic bond	

Enzymatic treatment in fruit juice processing is employed to improve juice yield, quality, and stability by degrading cell walls, pectin, starch, and suspended solids (Singh et al., 2019). This process enhanced total soluble solids (TSS), improved liquefaction, decreased viscosity and turbidity, and allowed better retention of nutritional value and colour (Danalache et al., 2018; Phung et al., 2019; Ramadan, 2019; Singh et al., 2019). Commercial pectic enzymes, such as pectinase, mainly target pectin, a complex polysaccharide containing chains of galacturonic acid units, rhamnose, galactose, and arabinose (Lara-Espinoza et al., 2018; Toy et al., 2022). As reported by Toy et al. (2022), pectinase is classified into three groups: pectin methylesterase (PME), protopectinase, and depolymerising enzymes. PME initiates pectin breakdown by converting it into pectic acid through de-esterification, while protopectinase breaks down protopectin into highly polymerised pectin. Depolymerising enzymes, in contrast, hydrolyse glycosidic linkages in pectin molecules, breaking them into simpler components (Toy et al., 2022). The enzymatic breakdown of pectin reduces its water-binding capacity, thereby releasing free water in the system and increasing juice yield (Sharma et al., 2017).

Cellulase enzymes break down cellulose, a structural carbohydrate that is mainly composed of complex sugars that protect and provide structure to the plant, into glucose. The cellulose fibres are surrounded by other structural biopolymers, primarily hemicellulose and lignin, forming a matrix (Danalache et al., 2018; Sharma et al., 2014).

Cellulase, a complex enzyme system, is composed of three enzymes: endo-β-1,4-glucanases (EG), exo-β-1,4-cellobiohydrolases (CBH), and β-glucosidases (BG) (Singh et al., 2019). The process involves the intricate hydrolysis of β-1,4-D-glycosidic bonds in cellulose and β-D-glucans by EG, followed by the precise hydrolysis of β-1,4-D-glycosidic bonds from the non-reducing ends by CBH, releasing cellobiose units (Toy et al., 2022). BG further hydrolyses the cellobiose into glucose monomers, facilitating the breakdown of cellulose into simple sugars (glucose) (Singh et al., 2019).

Hemicellulose is categorised based on sugar residues, including D-galactan, D-mannan, and D-xylan (Toy et al., 2022). This plant material is firmly attached to cellulose through hydrogen bonds and covalently linked to lignin (García, 2018). Toy et al. (2022) reported that the incorporation of hemicellulase is essential for the complete degradation of cell wall polysaccharides, as it facilitates the breakdown of tightly connected cellulose. This enzymatic process involves breaking hemicellulose into smaller oligosaccharides and monosaccharides.

Often, prioritising common enzymes such as pectinase, hemicellulose, and cellulase over complex, specific enzymes was due to several reasons. Firstly, direct targeting of enzymes is prioritised. For example, pectinase, encompassing polygalacturonase, pectin lyase and pectin esterase activities, is usually used, they have a direct effect on pectin, whereas pectin lyase acts only on highly esterified pectin with specific high methoxyl

matrices such as apples (Hossain & Ahmed, 2023). The cost of formulation was also taken into consideration, as common enzymes are much more cost-effective and widely available than specific enzymes, which may later increase the cost of production for the same amount of outcome.

OPTIMISATION METHOD USED FOR ENZYMATIC TREATMENT

The high cost of enzyme-based juice extraction, primarily due to the expensive nature of enzymes, highlights the need for process optimisation based on juice quality parameters (Kunkulol et al., 2018a). This requires careful planning and consideration of cost, operational efficiency, consumer preferences, and regulatory compliance (Kunkulol et al., 2018a). By optimising process variables, it is possible to reduce extraction time and cost while preserving its nutritional value, making it more viable for the food industry (Sonawane et al., 2020). Generally, the optimisation of food processing using the One-Factor-At-a-Time (OFAT) method, where only one variable was adjusted at a time while keeping other variables constant (Bensi et al., 2020). However, this approach is limited, as it does not consider the interaction among variables and does not provide a comprehensive understanding of their combined effects (Bensi et al., 2020).

Enzymatic treatment of fruit juice often applies the response surface methodology (RSM) method. RSM is a collection of statistical and mathematical techniques designed to develop a polynomial model to predict and enhance the behaviour of a dataset, particularly in scenarios where multiple variables influence the outcomes (Bensi et al., 2020; Kunkulol et al., 2018b). This approach is particularly effective in optimising, designing, developing, and enhancing processes affected by various factors, making it a powerful tool in the food industry (Kunkulol et al., 2018a; Montgomery, 2017). In fruit juice production, RSM demonstrates its precision by optimising parameters such as enzyme concentration, incubation time, and temperature (Pradhan et al., 2020).

The enzymatic treatment process mainly affects functional properties, such as antioxidant activity, total phenolic content, and colour (Kovács et al., 2024). This treatment can improve the functional properties by increasing the release of bioactive compounds from the fruit matrix. However, navigating this complexity is challenging, as several factors can influence enzyme activity during the enzymatic treatment of fruit juices, including enzyme concentration, incubation temperature and time, pH, and the type of enzyme used (Phung et al., 2019; Verma et al., 2018). By systematically varying these factors within a defined range and analysing the responses, RSM reliably determines the optimal conditions for enzymatic treatment to achieve the desired outcomes (Montgomery, 2017). This systematic approach allows researchers to identify the optimal combination of parameters that result in the highest juice yield, clarity, and final product quality (Kumar & Singh, 2019).

EFFECT OF OPTIMISED ENZYMATIC TREATMENT ON PHYSICOCHEMICAL PROPERTIES OF FRUIT JUICE

The use of cell wall-degrading enzymes such as pectinase, cellulase and hemicellulase has been studied to improve the extraction of fruit juice. Enzymatic treatment is an appropriate tool in addressing issues relating to pectin in fruit juice, especially as pectin is the major contributor to lower yield, high viscosity, cloudy juice and also the difficulties in the filtration process. As optimisation comes in handy, both research and industry can benefit from this process, including economic advantages and lower production costs and labour, all while also maintaining and improving the quality of fruit juice (Vivek et al., 2019). The research optimises various process variables used to attain the highest possible yield and quality of extracted juice, which includes enzyme concentrations, incubation time and temperature. A summary of optimised conditions to maximise juice yield of different tropical fruits is summarised in Table 2.

Juice Yield

A study by Singh et al. (2022) on the production of sapodilla juice from enzymatic treatment proved that pectinase has successfully enhanced the juice yield to 62.08%, increasing by 27.08% from untreated sapodilla juice (35%). Optimised enzymatic conditions were achieved at 0.12% enzyme concentration, 42.02 °C incubation time for 167.82 minutes. While enzyme concentration might have a significant role in maximising juice production in sapodilla fruit (Singh et al., 2022), optimising all parameters, including the incubation time and temperature, is equally important for efficient enzymatic activity throughout the process. Sharma et al. (2017) highlighted that juice yield is influenced by different types of fruits, cell walls and other fruit components, as well as enzymatic activity, which affect the facilitation of juice release. For instance, in the enzymatic treatment of guava juice, Kumar & Singh (2019) reported a 17.3% increase in juice yield from an optimised multi-enzyme condition using 0.96% pectinase, 0.57% cellulase, and 0.77% hemicellulase for 99 minutes of incubation time at 55 °C. This finding proved that it is important to determine the optimal enzymatic treatment conditions for fruit juice production to achieve high processing efficiency and quality (Sharma et al., 2017).

Viscosity

Viscosity is an important response variable in multiple enzymes treated in fruit juice. High viscosity in fruit juice poses a filtration problem, mainly attributed to pectin, cellulose, and hemicellulose in the fruit. Consequently, there is a consumer demand for clarified juice with lower viscosity to mitigate these issues (Vani et al., 2020). At an optimal enzyme concentration, the enzyme can effectively break down pectin, increasing juice yield and reducing the viscosity of the fruit juice, making it easier to filter. A summary of the enzymatic treatment in the viscosity reduction of fruit juice is provided in Table 3.

Table 2
Effect of optimised enzymatic conditions on juice yield of different tropical fruits

Fruit	Enzyme	Conditions			Juice Yield (%)	Ref
		Enzyme Concentration	Incubation Time	Temperature (°C)		
Guava	Pectinase Cellulase Hemicellulase	0.96% 0.57% 0.77%	99 min	55	64.7	Kumar & Singh (2019)
Sapodilla	Pectinase	0.12%	167.83 min	42.02	62.08	Singh et al. (2022)
Bael Fruit	Pectinase	0.22%	6.35 hours	46.20	56.66	Sonawane et al. (2020)
Watermelon	Pectinase	0.09% w/w	117.45 min	46.90	86.27	Saxena et al. (2014)
Passion Fruit	Pectinase	249.58 ppm	60 min	49.73	0.686 mL/g	Phung et al. (2019)
Chironji (*Buchanania Lanzan*) Fruit	Pectinase	0.08% w/w	99.5 min	45.8	70.73	Pradhan et al. (2020)
Jackfruit (*Koozha*)	Pectinase	0.5% v/v	2.75 hour	42.3	82	Bensi et al. (2020)
Soursop (*Annona muricata* L.)	Pectinase	0.04% w/w	172 min	42.9	75.20	Makebe et al. (2020)

Table 3
Effect of optimised enzymatic conditions on the viscosity of different tropical fruits

Sample	Enzyme	Conditions			Viscosity	Ref
		Enzyme Concentration	Incubation Time	Temperature (°C)		
Cashew Apple	Cellulase	0.091%	27.30 min	34.29	1.53 cP	Abdullah et al. (2021)
	Tannase	0.08%	20 min	30	1.47 cP	
Sapodilla	Pectinase	0.12%	167.83 min	42.02	9.1 cP	Singh et al. (2022)
Wild Watermelon (*Citrullus lanatus*)	Pectinase	0.15% w/w	60 min	60	1.96 cP	Mamabolo & Tabit (2023)
Guava	Pectinase	0.121% v/v	95 min	32.7	486.66 cP	Vani et al. (2020)
Bael Fruit	Pectinase	0.22%	6.35 hours	46.20	2.02 cP	Sonawane et al. (2020)
Passion Fruit	Pectinase	249.58 ppm	60 min	49.73	5 cP	Phung et al. (2019)
Banana	Cellulase	0.34%	29.33 min	36.5	101.14 mPa.s	Handique et al. (2019)
	Pectinase	0.35%				
Casimiroa (*Casimiroa Edulis*)	α-amylase (*Aspergillus Oryzae*)	0.2%	62.74 min	44.01	203.76 mPa.s	Genanew et al. (2022)

Abdullah et al. (2021) reported that enzymatic treatment using cellulase and tannase resulted in a low viscosity of cashew apple juice. In cellulase-treated juice, the enzyme concentration, incubation time, and temperature negatively affected the viscosity of the juice, indicating a consistent inverse relationship between all three parameters and viscosity. Although there was no significant effect between parameters interacting with the viscosity, this response's reduction was mainly attributed to the increase in enzyme concentration. This relationship contrasts with the positive effect of enzyme concentration on juice yield. Regardless of this trend, enzyme concentration, incubation time, and temperature still have a positive parabolic effect on viscosity. Tannase-treated cashew apple juice, on the other hand, shows a significantly negative effect (linear) of incubation time and enzyme concentration, with a non-significant ($p > 0.05$) effect of temperature towards the viscosity of cashew apple juice. The viscosity reduction in fruit juice production was due to the cell wall-degrading enzyme and the low water-holding capacity of the soluble polysaccharide in the cell wall, which released more water into the system (Abdullah et al., 2021). A similar finding was reported in pectinase-treated sapodilla juice, which reduced the viscosity from 9.1cP of an untreated control sample to 4.81cP of an optimised sample (Singh et al., 2022).

Clarity and Turbidity

Enzyme treatment is an important process in fruit juice production as it aims to break down pectin and other complex carbohydrates present in fruits, particularly before juicing. This process is critical as it enhances the clarity and visual appeal of the juice for consumers. For instance, Kunkulol et al. (2018b) demonstrated that enzymatic treatment of mango juice with 0.15% amylase and 0.40% pectinase at 150 min incubation time and 55 °C resulted in the highest level of clarification with 93.54%.

Meanwhile, the lowest clarity of mango juice (84.75%) was observed at 0.15% amylase and 0.20% pectinase for 90 min of incubation at 45 °C. Therefore, optimising all parameters ensures that the clarification process of fruit juice is efficient, cost-effective, and high in quality. This result is also consistent with Singh et al. (2022), who reported that enzymatic treatment affects the transmittance percentage of the sapodilla juice due to pectin degradation by pectinase.

Turbidity and clarity are often inversely related, indicating that a high turbidity value of fruit juice signifies that the juice is low in clarity. Hence, a more clarified yet less turbid fruit juice is preferable in juice production compared to a less clarified and highly turbid juice. For example, in wild watermelon juice production (Mamabolo & Tabit, 2023), enzymatic treatment has successfully reduced the turbidity of wild watermelon juice from 3208.5 NTU to 1800 NTU under optimised conditions of 0.15% w/w of pectinase and incubation for 60 min at 60 °C. The optimisation process shows a positive linear relationship with incubation time and a negative linear relationship with temperature

towards turbidity, with a non-significant effect from enzyme concentrations. These findings reflect the significant quadratic effect of both incubation time and temperature. Although enzyme concentration was expected to significantly impact the pectin hydrolysis, possible flocculation and sedimentation of pectin units may occur (Saxena et al., 2014). This trend might differ in other fruit juice production, as reported in banana, cashew apple, and guava juice, as simplified in Table 4.

Total Soluble Solid (TSS)

The increase of total soluble solids (TSS) in fruit juice has been influenced by enzymatic treatment due to the breakdown of pectin, cellulose, and hemicellulose, releasing more soluble sugars into the juice. A summary of the effect of optimised enzymatic conditions on total soluble solids of different tropical fruits is provided in Table 5. In the literature, it was shown that there was a significant difference between untreated (4.27 °Brix) and optimised enzyme-treated sugar palm juice (4.93 °Brix) (Arsad et al., 2015). This finding was similar to Handique et al. (2019), where the reduced TSS value of banana juice was found to be due to increased incubation time, cellulase, pectinase concentration and temperature. The combination of both optimised cellulase (0.34%) and pectinase (0.35%) concentration, with 29.33 minutes of incubation and a temperature of 36.5 °C in banana juice, increased the TSS value to 12.10 °Brix. Conversion of pectin by pectinolytic enzymes and the action of cellulase on cellulose caused a higher tissue degradation that may lead to the production of more soluble sugars (Arsad et al., 2015).

EFFECT OF OPTIMISED ENZYMATIC TREATMENT ON FUNCTIONAL PROPERTIES OF FRUIT JUICE

Antioxidant Activity

In recent years, there has been an increasing interest in antioxidant properties in fruit juice, attributed to its potential health benefits, tackling oxidative stress and lowering the risk of chronic disease. Improving and maintaining the antioxidant activity of fruit juice is crucial, as it is affected by temperature, processing conditions, and chemical composition (Grobelna et al., 2019). In noni juice production, the enzymatic treatment, assisted with an ultrasonic technique, increased antioxidant activity compared to the control due to cell wall degradation (Wang et al., 2023).

Similarly, Vivek et al., (2019) reported that sohiong fruit, a temperate fruit, also increased antioxidant activity in enzyme-ultrasonic assisted treatment (86.33%) from 82.67% of the control sample at the optimised conditions of pectinase concentration (0.05%), incubation time (57.44 minutes), temperature (45 °C), and ultrasound amplitude (90%) with ultrasound treatment time (9.95 minutes).

Table 4
Effect of optimised enzymatic conditions on clarity and turbidity of different tropical fruits

Sample	Enzyme	Conditions			Clarity	Turbidity	Ref
		Concentration	Time	Temperature (°C)			
Star Fruit (B10)	Pectinex Ultra SP-L (*Aspergillus niger)*	0.10%	20 min	30	0.864 Abs	0.88 NTU	Mazlina et al. (2008)
Star Fruit (B11)		0.1%	30 min	30	0.964 Abs	0.996 NTU	
Jamun Fruit	Pectinase (*Aspergillus aculeatus*)	0.05%	80 min	44	74.39 %T	32.21 NTU	Ghosh et al. (2016)
Sapodilla	Pectinase	0.12%	167.83 min	42.02	0.72 %T	13.73 NTU	Singh et al. (2022)
Guava	Pectinase	0.96%	99 min	55	-	17 NTU	Kumar et al. (2019)
	Cellulase	0.57%					
	Hemicellulase	0.77%					
Sugar Palm	Pectinex Ultra SP-L	0.05%	60 min	45	0.48 OD	-	Arsad et al. (2015)
	Cellulase (*Aspergillus niger)*	0.05%			0.49 OD	-	
	Pectinase + Cellulase	0.05% + 0.05%			0.39 OD	-	

Table 5
Effect of optimised enzymatic conditions on total soluble solids of different tropical fruits

Sample	Enzyme	Conditions			TSS (°Brix)	Ref
		Concentration	Time	Temperature (°C)		
Casimiroa (*Casimiroa Edulis*)	α-amylase (*Aspergillus Oryzae*)	0.2%	62.74 min	44.01	16.93	Genanew et al. (2022)
Soursop (*Annona muricata* L.)	Pectinase	0.04% w/w	172 min	42.9	12.22	Makebe et al. (2020)
Passion Fruit	Pectinase	249.58 ppm	60 min	49.73	12.3	Phung et al. (2019)
Palm (*Borassus flabellifer*)	Pectinase (*Aspergillus niger*)	0.33% w/w	171.32 min	33.75	10.2	Mohanty et al. (2018)
	Cellulase (*Trichoderma viride*)	0.39% w/w	108.5 min	60	10.3	
	Pectinase + Cellulase	1:0.75 w/w	120 min	55	12.8	
Watermelon	Pectinase	0.09% w/w	117.45 min	46.90	8.7	Saxena et al. (2014)

The assisted ultrasound treatment mentioned is to show the different relevant approaches made in enzymatic treatment, often to assist in promoting more enzyme accessibility to the polysaccharide cell wall, as assisted through the influence of structural disruption by the ultrasound. The addition of assisted treatment was also sometimes chosen to enable shorter processing times and lower dosage of enzymes used while maintaining the same achievement of enzymatic treatment. However, whether the combined treatment yields a significantly better result when compared to enzyme treatment only depends on its ability not to hinder the system or affect antioxidant stability. The use of assisted ultrasound was not commonly found within the industry scale of production as additional ultrasound-assisted performance often depends on additional parameters, including power, frequency and juice matrix, complicating the already standardised protocols of enzymatic treatment (Alshehhi et al., 2023).

Consequently, enzymatic treatment alone might have an inverse effect on antioxidant activity in other fruit juice production, as different types of fruit have different cellular structures and nature in their bioactive compounds. For example, in pear juice, the DPPH (19.30%) and ABTS (18.70 mg catechin/100 g) values are lower in enzyme-treated samples than in untreated samples (20.30% and 18.91 mg catechin/100 g) (Grobelna et al., 2019). The contradictory effects of enzymatic treatment towards the antioxidant activity across different types of tropical fruit can be attributed to the mechanistic factors of their matrices. For example, as enzymatic hydrolysis can actually enhance the extractability of phenolic and antioxidant compounds through the breakdown of the cell wall binding these compounds, enzymatic treatment can also lead to possible oxidative loss of sensitive antioxidants such as phenolic acids and ascorbic acids as it continues to be exposed to oxygen. This later has resulted in the reduction of measured radical scavenging activity (Wang et al., 2023). According to Gani et al. (2021), the reduction in antioxidant activity in enzyme-treated pear juice might be associated with the possible occurrence of bioactive compounds to oxidise during heating, proving that antioxidants are highly temperature dependent. Therefore, enzymatic treatment may enhance antioxidant activity in some fruits, but parameter optimisation remains important in enhancing and reducing the possible loss of bioactive compounds during processing.

Another important aspect to be crucially observed is the effect of excessive treatment on phenolic and antioxidant compounds. The breakdown of cell wall and polysaccharide chains during enzymatic treatment allows natural oxidative enzymes such as polyphenol oxidase (PPO) and peroxidase (POD) to be released within the system. This was found to increase the oxygen transport, catalysing the conversion of phenolics into quinones, forming brown pigments and reducing radical scavenging capacity (Singh et al., 2018). L-ascorbic and L-dehydroascorbic acids are the two main compounds of vitamin C with antioxidant activity, where their stability depends on the amount of oxygen it's being exposed to, where

the degradation of these acids is due to the oxidation process (Pérez-Lamela et al., 2021). Prolonged incubation leads to more or excessive exposure towards oxygen, leading to the degradation of several desirable antioxidants.

Total Phenolic Content

Phenolic content is important as it is closely related to the sensory quality, pigment accumulation for appearance, and antioxidant ability of fruit juice (Wang et al., 2023). Table 6 demonstrates a few studies conducted on tropical fruit juice using various types of enzymes and conditions. Similar to antioxidant activity, phenolic compounds are also susceptible to technological processes, affecting the astringency and quality of fruit juice.

The phenolic content in fruit-based products plays a significant role in health, protecting the body against possible coronary heart disease, cancers and protecting vitamin C in fruit juice from oxidative degradation (Nur 'Aliaa et al., 2011). Additionally, Nur 'Aliaa et al. (2011) showed two optimised conditions suitable for enhancing total phenolic contents in red pitaya juice by using 0.10% Pectinex Ultra SP-L (with 45 minutes incubation time, and temperature of 40°C), and 0.09% Pectinex CLEAR (with 85 minutes incubation time at 45 °C).

Table 6
Effect of optimised enzymatic conditions on total phenolic content of different tropical fruits

Sample	Enzyme	Conditions			TPC	Ref
		Concentration	Time	Temperature (°C)		
Jamun Fruit	Pectinase (*Aspergillus aculeatus*)	0.05%	80 min	44	115.31 mg GAE/g	Ghosh et al. (2016)
Bael Fruit	Pectinase (*Aspergillus niger*)	0.22%	6.35 hours	46.20	56.66 mg/100mL	Sonawane et al. (2020)
Red Pitaya	Pectinex Ultra SP-L (*Aspergillus niger*)	0.10%	45 min	40	1.34 mg/100g	Nur 'Aliaa et al. (2011)
	Pectinex CLEAR (*Aspergillus aculeatus* and *Aspergillus niger*)	0.09%	82 min	46	1.26 mg/100g	
Casimiroa (*Casimiroa Edulis*)	α-amylase (*Aspergillus Oryzae*)	0.2%	62.74 min	44.01	45.09	Genanew et al. (2022)

The total phenols of optimised enzymatic treatment using Pectinex Ultra SP-L and Pectinex CLEAR were found at 1.34 mg/100g GAE and 1.26 mg/100g GAE, respectively, suggesting an increase from untreated red pitaya juice (1.24 mg/100g GAE) (Nur 'Aliaa et al., 2011). This trend is aligned with casimiroa juice, where optimised conditions of 0.2% enzyme concentration at 62.74 minutes and 44.01 °C increased the total phenolic content (25.74%) compared to the untreated casimiroa juice (Genanew et al., 2022). As enzymatic treatment was conducted under specific and mild conditions, such as controlled temperature and pH level, the likelihood of degradation of sensitive compounds can be minimised, as can be seen from the linear effect of both antioxidant activity and total phenolic content throughout enzymatic treatment on certain tropical fruits. This can denote that the bioactive compounds were not completely degraded under optimised treatment conditions. However, although Folin-Ciocalteu helped in measuring the improvement of extracted phenolics, more chromatographic studies were needed to analyse the total phenolic content, stability, along with the potential degradation of sensitive compounds throughout the enzymatic treatment.

Maintaining the optimal temperature condition across enzymatic treatment is a crucial factor to balance the catalytic effectiveness and the thermal stability of the bioactive compounds. Enzyme activity normally increases along with the increase of temperature to a certain optimal point, improving the hydrolysis or degradation of polysaccharide cell wall, hence enhancing the extractability of bound bioactive compounds into the juice matrix (Palos- Hernández et al., 2024). On the other hand, treatment beyond optimal temperatures may hinder the stability of enzymes, thus causing the denaturation of enzyme and accelerated degradation of heat sensitive antioxidants (like ascorbic acid) and isomerisation of certain phenolic compounds (flavonoids and vitamins), leading to the decrease of antioxidant capacity despite the increased extractability observed (Bastos et al., 2025; Palos-Hernández et al., 2024).

Although this review does not focus on the influence of enzymatic treatment towards the changes of sensory attributes, we acknowledge that this treatment indeed has a huge impact on its influence. Enzyme hydrolysis may affect sensory qualities in addition to efficient extraction of volatile chemicals. Enzymatic treatment substantially modifies the sensory properties through releasing glycosidically bound volatiles that are responsible for improving aroma, enhancing the concentration of phenolics linked to produce bitterness, and also speeding up the loss of delicate volatiles under prolonged incubation, excessive thermal pressure and possible oxygen exposure, which may alter the lipid profile of such fruit juice (de Carvalho et al., 2015).

CONCLUSION

Optimised enzymatic treatment has enhanced various quality attributes of tropical fruit juices, such as juice yield, viscosity, clarity, turbidity, total soluble solids (TSS), antioxidant

activity and total phenolic content (TPC). Enzymatic treatment using pectinase, cellulase, and hemicellulase in degrading cell wall structures and releasing valuable compounds was seen to have a significant effect. Despite that, excessive or prolonged application of this treatment may result in undesirable effects. Therefore, optimising the enzymatic treatment conditions, including enzyme concentration, incubation time, temperature, and any other variable used in tropical fruit juice production, is important. It should be noted that different types of fruit may have different enzymatic treatment conditions depending on their pectin, cellulose, and hemicellulose content. Even so, industry can still use this as a production baseline and guidance in understanding the need for optimisation and enzymatic treatment of each tropical fruit in juice production, in the hope of bringing efficiencies in larger industrial cases.

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ETHICAL STATEMENT

Not applicable.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Abscisic Acid Exogenous Spraying to Improve Sorghum (*Sorghum bicolor* (L.) Moench) Genotypes for Tolerance to Abiotic Stress

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ABSTRACT

Climate change poses significant challenges to agricultural production, particularly in semi-arid and arid regions, characterised by high temperatures and frequent drought conditions. Choosing appropriate crop genotypes and effective agronomical management practices for tolerance to abiotic stress becomes crucial for sustaining crop productivity under these conditions. This experiment aimed to determine the effect of exogenous abscisic acid spraying (distilled water only, 9 and 12 ppm) as the main factor on the performance of four sorghum genotypes (Bohooth, Giza, Al-Khair, and Warkaa) as sub-factors by using a split-plot design in RCBD repeated thrice. The study results showed that the interaction of abscisic acid spray at 12 ppm with the Giza and Al-Khair genotypes outperformed the control, recording the highest averages in most of the sorghum morphological indices. The abscisic acid spray had a significant positive impact on the sorghum genotypes, significantly enhancing growth parameters and grain yield, especially at 12 ppm concentrations. These findings suggest that ABA spraying has a potential priming influence to enhance sorghum grain filling capacity under heat stress during the grain filling stage, particularly by inhibiting the reactive oxygen system and increasing starch synthetase activities in sorghum grain.

Keywords: Abscisic acid, abiotic stress, genotypes, sorghum growth, yield component

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INTRODUCTION

Sorghum bicolour (L.) Moench belongs to the Poaceae family, globally positioned fifth among grain crops in terms of cultivated area and production, after wheat, rice, barley, and maize (Maaruf & Raheem, 2024). Sorghum grains are used in human and animal nutrition in poor countries.

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They are used as human food when mixed with wheat flour at a rate of 50%. In developed countries, such as the United States, approximately 90% of the maize grains are used in human food industries, such as starch and its derivatives (Mouhamad & Fadhil, 2025) and are a crucial forage and biofuel energy crop (Yang et al., 2025).

Plant growth regulators are non-nutritive organic compounds that encourage, inhibit or modify plant biological processes and are formed at very low concentrations (Taha et al., 2025). Abscisic acid does not directly participate in cell division and expansion, but it works indirectly to regulate the growth of plants under the influence of stress by controlling the transpiration process, controlling the stomata opening and closing. In addition, other important effects that would keep the plant under the influence of biotic and abiotic stresses in good condition (Ali et al., 2020).

Climate change has significantly influenced world agricultural production, posing considerable challenges to food security, particularly in semi-arid and arid regions vulnerable to high temperatures and drought stress (IPCC, 2023). Salah Al-Din governorate in Central Iraq is highly susceptible to extreme heat events, with temperatures frequently reaching 45°C or more in July and August on some days with reduced relative humidity and high evaporation amount. These abiotic stresses severely affect crucial plant growth stages, notably flowering and grain formation stages, causing substantial yield reductions. Sorghum crop is recognised for its resilience to high-temperature stress and limited water availability, making it an essential crop for sustaining agricultural production under semi-arid conditions (Pradhan et al., 2022). A 26-34 °C air temperature is optimum for sorghum at the vegetative duration and 25-28 °C at the reproductive duration, as stated in Prasad et al. (2015). Remarkably high temperatures result in a decrease in yield. With increased day and night temperatures, flower initiation and floral primordial development are delayed. Spraying with abscisic acid is critical in minimising the adverse impacts of heat stress by improving the reactive oxygen system-scavenging ability to discourage the overaccumulation of reactive oxygen system and alleviate membrane injuries. Superinduction of the stress tolerance-related expresion of genes under high temperature stress suggests that abscisic acid has a possible priming impact on the gene expression regulatory network for coping with heat stress factors (Liu et al., 2023). Additionally, with favourable climatic conditions, the crop genotypes significantly influence crop performance by promoting vegetative growth, reproductive development, and enhancing plant resistance to environmental stresses.

Climate change compels researchers to be concerned about food security. Hence, studying the phytohormones will assist in elucidating their accurate place and developing climate-resilient and high-yielding crop cultivars. The ABA role was confirmed against different stresses, chiefly abiotic stresses and in various physiological processes in crops. Abscisic acid (ABA) shares a critical part of its numerous functions during the fruit

development and ripening phases in crop plants (Gupta et al., 2022). Adverse abiotic ecological conditions, such as maximal temperature, severely affect crop growth and productivity. When crops are exposed to various ecological conditions, the ABA level increases significantly. Hence, for the plants' appropriate growth and development, the maintenance of a basal ABA level is paramount (Ali et al., 2020).

The ABA phytohormone has high importance in the development of mechanisms and various stress responses to the environment when plants experience certain biotic or abiotic stresses, including extreme temperatures (Rehman et al., 2022). The extremely high temperatures, which result in heat stress, have become an intensely restrictive factor for crop production in the world. The sorghum growth duration largely occurs during the hot months of the summer season, resulting in intense heat stress that affects its growth and yield. Thus, a sorghum grain yield improvement under heat-stress environments is a major challenge for farmers in Iraq. Sorghum in the reproductive growth period is sensitive to heat stress because it causes a reduction in pollen vitality and affects the meiosis progression and development of sorghum heads, which causes a reduction in sorghum grain spikelets filled and a decrease in yield component traits. The grain-filling phase is the crucial stage for starch synthesis, grain filling and yield formation. Therefore, it is crucial to enhance the thermotolerance of sorghum during the grain-filling stage to achieve the highest possible yield under heat stress conditions. Abscisic acid, a phytohormone, is an efficient and effective way for crops to adapt to heat stress conditions. ABA is found to play a vital role in enabling rice to adapt to heat stress, conferring possibly improved stress tolerance on grains or seedlings pre-treated with abscisic acid, and its response to heat stress, and exogenous abscisic acid enhances thermo tolerance and yield in rice in heat stress conditions. Pre-treatment with abscisic acid has been shown to considerably toughen the abscisic acid signal in rice at heat stress (Liu et al., 2023).

Attention should be given to high-yielding Sorghum varieties, particularly newly introduced varieties, while taking into account the application of scientifically sound crop maintenance processes and adopting the necessary methods to achieve this using modern scientific methods and the introduction of new varieties. In the country, it is compared with the existing varieties, and then the best varieties are chosen to increase the productivity of this crop and improve its quality (Raheem et al., 2023). The differences in genetic composition among varieties show clear differences in growth and production characteristics (AL-Azee et al. 2023), and the interaction of genetic factors with the environment shows its effect on the phenotypic and production characteristics. All of these factors participate in determining the increase or decrease of these characteristics (Al-Rifai, 2017). Azawi (2023) observed during her study on five Sorghum cultivars (J, Giza, Enqaz, Rabeh and Li) that the Li cultivar was superior in leaf area, 4086.61 cm^2, while the J cultivar was superior in plant height, 177.48 cm. The Giza cultivar was superior

in most growth traits, including the number of leaves and chlorophyll a and b content; it produced 16.43 leaves plant^{-1}, 23.40-µg g^{-1} and 19.83 µg g$^{-1,}$ respectively.

Based on available information, ABA has not previously been used to prevent sorghum crop stress caused by high temperatures during early sowing. Therefore, the study aims to evaluate its effectiveness as an avoidance strategy by spraying with different concentrations to mitigate the effects of high temperatures in the field. This includes protecting plant growth, especially the reproductive organs during flowering and grain formation after pollination, to maximise grain yield. Abscisic acid is a growth regulator that is found in plants under various stresses. Unsuitable high temperatures at the sorghum crop flowering stage can significantly reduce productivity. Thus, this work seeks to assess the influence of ABA spraying in decreasing the negative heat stress effect and increasing sorghum grain production.

MATERIALS AND METHODS

Description of Experiment Location

To achieve the above goal, a field experiment was conducted on the 2024 summer growing season in the Al-Alam district of Salah Al-Din Governorate, located at 43.63 °E and 34.65 °N with 98 m MSL. Table 1 shows the climate elements description in Salah Al-Din Governorate during the experiment period, and Table 2 shows the most important experiment field which is physical and chemical soil characteristics at a depth of 30 cm.

Experimental Design and Studied Factors

The first factor included three levels of abscisic acid placed in the main plots, which were as follows: spraying with distilled water only, spraying with abscisic acid at 9 and 12 ppm, sprayed in two stages, at the eight-leaf stage and the pre-flowering stage. Abscisic acid solution was prepared according to Al-Jawaheri & Alogaidi's (2020) description. The brand of abscisic acid is A4906, which was produced by American Sigma-Aldrich Company in powder form and has a ≥ 98% (HPLC) assay. The second factor included four Sorghum genotypes (Bohooth, Giza, Al-Khair, and Warkaa) placed in subplots. The source of the Sorghum genotypes' seed is Syria (Bohooth), Egypt (Giza) and Iraq (Al-Khair and Warkaa). The Randomised Complete Block in a split-plot design was used to carry out the field experiment and repeated three times.

Each replicate was divided into three main plots where abscisic acid was placed, and each main plot was divided into four subplots where the four Sorghum genotypes were planted. Each replication contained 12 experimental units, which included four lines of 3 m length, at a space between each line of 0.70 m and between each hole of 0.20 m. The field was fertilised with an amount of 200 kg N ha^{-1} added in three equal doses at planting,

Table 1
The experiment location's climatic conditions (monthly averages)

Months	Max. Temp. (°C)	Min. Temp. (°C)	Avg. Temp. (°C)	Max. RH (%)	Min. RH (%)	Avg. RH (%)	ET (mm)
Jul.	43.55	29.75	36.65	29.33	8.30	18.82	10.53
Aug.	45.39	28.85	37.12	29.93	7.57	18.66	8.12
Sept.	40.20	24.73	32.47	35.31	11.90	23.58	6.56
Oct.	32.38	16.65	24.52	39.56	10.45	25.01	4.08
Nov.	22.59	10.42	16.51	95.40	37.30	65.50	1.89
Dec.	17.39	4.10	10.75	87.83	34.85	61.34	1.45

Note. Max. - Maximum; Min. - Minimum; Avg. - Average; Temp. - Temperature; RH - Relative Humidity; ET - Evaporation Amount

*Source: Agricultural Meteorology Centre, Salah al-Din Governorate - Tikrit Station, Ministry of Agriculture, Iraq (2024)

Table 2
Soil physical and chemical properties

Properties	pH	EC	Avail. N	Avail. P	Avail. K	O.M.	Sand	Clay	Silt	Texture
Values	7.3	1.71	6.5	9.7	100.3	0.7	494.20	160.30	345.50	Sandy
Measurement Unit	-	ds m^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	%	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	loam

Note. Soil samples were analysed at the Integrated Laboratories of the Department of Soil Resources and Water Sciences - College of Agriculture - Tikrit University, Salah al-Din, Iraq

during the elongation stage and the beginning of flowering. Phosphate 100 kg P_2O_5 ha^{-1} and potassium 100 kg K_2O ha^{-1} fertiliser were added in one dose before planting. The seeds were planted on July 1, at a depth of 2-3 cm, with 2-3 seeds put in each hole. The patching process was carried out one week after germination, and thinning was carried out three weeks after planting. One plant is left in each hole, and weeds are controlled according to need.

Data Collection

Data was collected on indices of plant height (cm) from the soil surface to the inflorescence node, plant leaves (leaf $plant^{-1}$) calculated at the head's appearance stage, plant leaf area (cm^2) determined according to Elsahookie and Cheyed (2014), and leaf chlorophyll content (mg g^{-1} fresh weight) estimated based on Mackinney's (1941) method. Head grains number (grains $head^{-1}$), head weight (g), 1000-grain weight (g), grain yield (t ha^{-1}), biological yield (t ha^{-1}) and harvest index (%) were calculated according to flowing formula: Grain yield (t h^{-1})/ Biological yield (t ha^{-1}) × 100 (Ashraa Kalee & Raheem, 2024).

Statistical Analysis

The F-test was done on the collected data for all studied indices according to the design used at $\alpha = 0.05$ and 0.01, then Duncan's multiple range test was applied for treatment means comparison at $\alpha = 0.05$ by the SAS program V9-2002 (SAS Institute, 2004).

RESULTS AND DISCUSSION

ANOVA-Table (Fisher's Test)

Analysis of variance for the studied indices in Table 3 shows that the effect of abscisic acid concentrations was significant at α 0.01 for all the studied indices except plant leaf number, which was significant at α 0.05; chlorophyll content and harvest index were not significant. The genotype effect was significant at α 0.01 for all the studied indices except the grain $head^{-1}$, which was not significant. The interaction effect of abscisic acid with genotypes was significant at α 0.01 in total grain yield, moreover, at α 0.05 in head weight, 1000-grain weight and harvest index, and not significant in plant height, plant leaves, leaves chlorophyll content, plant leaf area, grain $head^{-1}$ and biological yield.

Abscisic Acid (ABA)

The data presented in Table 4 show that exogenous foliar spraying of ABA on sorghum plants under high temperature stress conditions revealed significant benefits, especially at the highest concentration, 12 ppm, which was the superior concentration in achieving the highest values of all studied indices compared with the 9 ppm and control treatment.

Table 3
ANOVA-Table represented by means of squares (M.S.)

S.O.V.	R	A	R*A	B	A*B	Error	CV (%)
d. f.	2	2	4	3	6	18	
Plant Height (cm)	783.12	338.48**	2.44	1078.03**	14.46 N.S	20.50	2.9
Plant Leaves (Leaf Plant^{-1})	12.13	1.96*	2.31	5.10**	0.30 N.S	0.40	6.1
Leaves Chlorophyll Content (mg g^{-1} fresh weight)	133.80	9.61 N.S	11.67	112.97**	5.97 N.S	6.87	5.9
Plant Leaf Area (cm^2)	67400.84	120691.68**	235.81	353203.18**	2663.73 N.S	3583.13	1.0
Grains Head^{-1}	183642.69	60717.19**	6304.65	17848.66 N.S	3378.19 N.S	10057.85	4.2
Head Weight (g)	654.69	193068.77**	650.90	11608.62**	2510.70 *	757.31	4.7
1000-grain Weight (g)	4.75	96.58**	3.58	7.21**	4.21*	1.37	3.9
Grain Yield (t h^{-1})	0.549	6.31**	0.21	6.64**	1.54**	0.272	8.6
Biological Yield (t ha^{-1})	13.44	16.42**	0.165	5.72**	0.081 N.S	0.425	4.0
Harvest Index (%)	14.34	46.35 N.S	7.93	189.75**	53.58*	15.76	10.7

Note. ** = Significant at α 0.01; * = Significant at α 0.01; N. S = Not significant; R = Replication; A= Abscisic acid; and B = Genotypes; C.V. = coefficient of variation; Total d. f. = 35

The concentration of 12 ppm ABA was superior in plant height and gave an average of 161.50 cm, while the control treatment was the lowest average, reaching 151.07 cm. This effect may be due to ABA's role in regulating responses to stress, especially under high temperature conditions, which helps the plant manage abiotic stresses by reducing transpiration and enhancing water-use efficiency. Moreover, it helps maintain stomata closure, decreasing water loss during transpiration and enhancing water-use efficiency. In addition, improving chlorophyll content enhances the photosynthesis process. This, in turn, supports better growth leading to increased plant height, as the plant growth regulators increase the plant's height. These findings are in agreement with those stated about a positive effect of ABA spraying on increasing plant height by Gomaa et al. (2023) in rice, Zulfiqar et al. (2024) in wheat and Abd El-Hady et al. (2025) in maize as compared to the control treatment. With concentrations of 9 and 12 ppm ABA, the highest averages of 10.53 and 10.67 leaf plant^{-1} were recorded, respectively, with the control treatment recording the lowest plant leaves, reaching 9.91 leaf plant^{-1}.

This may be attributed to abscisic acid role in increasing plant height. These results indicate that ABA is effective in promoting leaf growth.

Kumar (2024) noticed similar results in wheat. The positive effect of ABA was clear on leaves' chlorophyll content; the concentration of 12 ppm of abscisic acid was superior, achieving an average of 45.67 mg gm^{-1} fresh weight, while the control treatment recorded the minimum average, reaching 43.97 mg gm^{-1} fresh weight. The increased chlorophyll content induced by ABA may be due to the protection of chloroplast pigments from harmful effects by increasing the activity of antioxidant enzymes. This was in line with those who found a positive role of ABA in leaf chlorophyll content enhancement in rice and wheat (Chen et al., 2022; Kumar, 2024). Similarly, the concentration of 12 ppm ABA was superior in plant leaf area, giving an average of 5880.53 cm^2 $plant^{-1}$, while the control treatment recorded the lowest average, reaching 5683.20 cm^2 $plant^{-1}$. The increase in the plant's leaf area is attributed to the ABA's role in increasing plant height and the number of leaves. The noticed results agree with those of Abd El-Hady et al. (2025).

In the case of the grain $head^{-1}$ indices, the concentration of 12 ppm ABA was also superior, yielding an average of 2472.67 grains $head^{-1}$, the lowest average recorded by the control treatment, reaching 2330.58 grains $head^{-1}$. This work highlighted the important role of increasing grain $head^{-1}$ and the importance of ABA in the flowering process.

These findings align with Hwaidi (2023), who found that ABA enhances flowers' resistance to heat and promotes high levels of pollination and double fertilisation. Moreover, it has a role in regulating the inner plant water content (Al-Jawaheri & Alogaidi, 2020).

Table 4
Abscisic acid effect on the studied indices

Indices	Abscisic Acid Concentrations (ppm)		
	0	9	12
Plant Height (cm)	151.07 c	158.02 b	161.50 a
Plant Leaves (Leaf $Plant^{-1}$)	9.91 b	10.53 a	10.67 a
Leaves Chlorophyll Content (mg g^{-1} Fresh Weight)	43.97 c	44.34 b	45.67 a
Plant Leaf Area (cm^2)	5683.20 c	5750.78 b	5880.53 a
Grains $Head^{-1}$	2330.58 b	2395.42 ab	2472.67 a
Head Weight (g)	481.08 c	558.08 b	728.92 a
1000-grain Weight (g)	27.75 b	29.67 b	33.33 a
Grain Yield (t h^{-1})	5.35 c	6.04 b	6.80 a
Biological Yield (t ha^{-1})	15.04 c	16.69 b	17.30 a
Harvest Index (%)	34.69 b	36.28 ab	39.35 a

Note. In the same row, similar letters do not differ significantly

In addition, spraying sorghum plants with abscisic acid contributed to increasing leaf area and stimulating carbon metabolism, which led to an increase in the grain head^{-1}. Al-Fayyadh and Hwaidi (2022) and Abd El-Hady et al. (2025) stated similar findings in maize. Concerning head weight, the concentration of 12 ppm ABA outperformed, yielding the heaviest head weight of 728.92 g, while the control treatment had a lighter head weight of 481.08 g. This is attributed to the fact that the abscisic acid treatment at this stage contributed to increased pollen fertility and activity, which led to an increase in grain head^{-1} and then an increase in head weight. A positive role of exogenous ABA was found in increasing sunflower capitulum weight (Hwaidi, 2023) and in rice panicle weight (Liu et al., 2023).

Moreover, the concentration of 12 ppm ABA is superior in 1000-grain weight, with an average of 33.33 g, while the lowest average recorded by the control treatment is 27.75 g. The spraying of ABA may encourage root growth, absorb more nutrients and water, and then support photosynthesis with the required nutrients, increasing the mobile carbon produced substances from metabolism processes that supply the key source of grain filling. Also, it facilitates the transfer of photosynthetic products from the source (leaves) to the sink (grains) and then increases grain weight. Similar findings were obtained by Al-Fayyadh and Hwaidi (2022) in maize and Wang et al. (2025) in rice.

For final yields, the concentration of 12 ppm of ABA was superior, yielding the highest grain yield of 6.80 t ha^{-1}, whereas the lowest yield was recorded by the control treatment of 5.35 t ha^{-1}. This may be attributed to the abscisic acid role in the yield components increasing (Table 4), which positively affected the total grain yield. These results were in line with those of Hwaidi (2023), Ahmed and Al-Tameemi (2025) for sunflowers, and Abd El-Hady et al. (2025) for maize. Similarly, the concentration of 12 ppm ABA outperformed in biological yield, yielding 17.30 t ha^{-1}, while the control treatment recorded the lowest 15.04 t ha^{-1}. This is due to the beneficial effect of abscisic acid in increasing crop growth and grain yield (Table 4). This information agrees with that indicated by Abd El-Hady et al. (2025). The concentration of 12 ppm ABA was superior in the harvest index with 39.35%, while the control treatment recorded the least at 34.69%. The superior harvest index with ABA spraying confirms its effective role in increasing the ratio of grain yield to total plant biological yield. This suggests that ABA may better improve overall plant efficiency and productivity. The higher seed yield resulted in improved harvest index (Table 4). Kumar (2024) and Zulfiqar et al. (2024) in wheat and Abd El-Hady et al. (2025) in maize, stated similar findings that correspond with our results.

Sorghum Genotypes

Significant differences among sorghum genotypes in all studied indices except the grain head^{-1} are shown in Table 5. The Bohooth genotype had significantly taller plants, 172.64 cm.

In contrast, the Giza and Warkaa genotypes had shorter plants, 149.04 and 149.93 cm, respectively. These differences in plant height among sorghum genotypes may be due to differences in their genotype in genetic makeup among the genotypes that determine the number of internodes and stem height, and thus, differences appear in the height of the plant and their ability to respond to environmental conditions, with their impact on increased cell division and elongation. These results are similar to the results of Azawi (2023), Maaruf & Raheem (2024) and Abas et al. (2025). The Bohooth, Giza, and Al-Khair genotypes outperformed in the highest plant leaves 11.00, 10.41 and 10.77 leaves plant^{-1}, respectively, while the Warkaa genotype had the lowest number of plant leaves 9.30 leaves plant^{-1}. The reason for the variation among genotypes may be due to differences in their genetic makeup. A particular composition may have the ability to express itself better under the influence of environmental factors, while other cultivars cannot. The plant's number of leaves are closely related to the amount of light captured and the CO_2 absorbed by the plants for photosynthesis. The more light received, the more photosynthesis is produced to support plant growth. Photosynthesis is considered one of the most significant methods to increase crop yield. The more leaves there are, the higher the photosynthesis. The result of photosynthesis is then translocated to other plant parts to produce high biomass (Kusumawardana et al., 2025). These results agreed with what was reported by Sharif et al. (2024) and Haque et al. (2025).

The Khair genotype was superior in chlorophyll content in leaves with 49.77 mg g^{-1} fresh weight. In contrast, the Warkaa genotype was the lowest, reaching 41.71 mg g^{-1} fresh weight.

Table 5
Sorghum genotypes' effect on the studied indices

Indices	Sorghum genotypes			
	Bohooth	**Giza**	**Al-Khair**	**Warkaa**
Plant Height (cm)	172.64 a	149.04 c	155.82 b	149.93 c
Plant Leaves (Leaf Plant^{-1})	11.00 a	10.41 a	10.77 a	9.30 b
Leaves Chlorophyll Content (mg g^{-1} Fresh Weight)	44.10 b	43.06 b	49.77 a	41.71 c
Plant Leaf Area (cm^2)	5683.76 c	6028.73 a	5808.21 b	5565.30 d
Grains Head^{-1}	2430.89 a	2437.56 a	2388.56 a	2341.22 a
Head Weight (g)	560.89 b	612.22 a	627.67 a	556.67 b
1000-grain Weight (g)	31.22 a	29.67 bc	30.78 ab	29.33 c
Grain Yield (t h^{-1})	5.03 c	6.90 a	6.62 a	5.70 b
Biological Yield (t ha^{-1})	16.52 b	17.20 a	16.38 b	15.28 c
Harvest Index (%)	30.51 b	40.29 a	40.18 a	37.45 a

Note. In the same row, similar letters do not differ significantly

The difference among genotypes in chlorophyll content in their leaves may be primarily due to genetic variation among them and their different responses to surrounding growth factors. These results agree with the results of Mouhamad and Fadhil (2025), who reported that the sorghum genotypes varied in leaf chlorophyll content. The Giza genotype excelled in plant leaf area with the highest value of 6028.73 cm^2 $plant^{-1}$, while the Warkaa genotype had the lowest value of 5565.30 cm^2 $plant^{-1}$. This difference among Sorghum genotypes is due to genetic differences in canopy structure and leaf development potential, with their differences in growth speed before reaching maximum leaf area. These results are consistent with those of Maaruf & Raheem (2024) and El-Sheikh et al (2025). The grain $head^{-1}$ was not significantly affected by Sorghum genotypes, whereas the Khair and Giza genotypes outperformed in head weight with 627.67 and 612.22 g, respectively; conversely, the Warkaa genotype had the lowest with 556.67 g. The difference between cultivars may be due to the genetic makeup of genotypes and the way each utilises the environmental conditions surrounding growth. The number of grains per head is linked to genetic factors, which are greatly affected by environmental conditions. This is similar to the results of Al-Shakarchy and Altaweel (2021). On the other hand, the Bohooth genotype outperformed with the high weight of 1000 grains, 31.22 g. In contrast, the Warkaa genotype had the lowest weight, reaching 29.33 g. This may be due to differences in the genetic material of each genotype that cause differences in the grain size and weight. Similar findings are reported by Raheem et al. (2024), Begna (2025) and Bajhir et al. (2025). The Khair and Giza genotypes produced maximum grain yield, 6.62 and 6.90 t ha^{-1}, respectively, whereas the Bohooth genotype yielded the lowest 5.03 t ha^{-1}. This was caused by differences in yield components among the sorghum genotypes (Table 5), consistent with the findings of Li et al. (2025) and Haque et al. (2025). Furthermore, in biological yield, the Giza genotype was superior with 17.20 t ha^{-1}. In contrast, the Warkaa genotype yielded the lowest at 15.28 t ha^{-1}. This happened because of the significant superiority of the Giza genotype in plant leaves, leaf area and grain yield (Table 5). This result is consistent with those of Raheem et al. (2023). Finally, only the Bohooth genotype differed significantly from the other genotypes by the lowest harvest index, 30.51%, while the other genotypes outperformed, with no significant differences among them. This is contributing to variations in genetic composition affecting grain yield and biological yield. A genotype may have a high yield, but its biological yield is low, which increases the harvest index. These results are followed by those observed by Raheem et al. (2023) in maize.

ABA × Genotypes Interaction

Table 6 shows that the Exogenous foliar spray of ABA exhibited significant differences due to the differences among sorghum genotypes. ABA improved the growth quality

and yield of sorghum genotypes in general. The interaction between 12 and 9 ppm ABA spraying and the Bohooth genotype was superior, giving the tallest plant a height of 176.00 cm and more plant leaves (11.67 leaves plant^{-1}). In contrast, the control treatment with the Warkaa genotype recorded the lowest height at 141.69 cm and plant leaf count of 9.10 leaves plant^{-1}. The average across the Al-Khair genotype, exogenous spraying of 12 ppm ABA increased the maximum leaf chlorophyll content, reaching 51.51 mg gm^{-1} FW; control conditions caused a reduction across the Warkaa genotype (40.31 mg gm^{-1} FW). The leaf area and grain head^{-1} of the Giza genotype showed an important increase at 12 ppm ABA spraying, reaching 6141.52 cm^2 and 2535.33 grain head^{-1}, respectively, relative to the control treatment of the Warkaa genotype, which recorded values of 5468.55 cm^2 and 2279.00 grain head^{-1}. On the other hand, a combination of 12 ppm ABA spraying with the Al-Khair and Giza genotypes showed a significant improvement in maximum head weights of 762.67 and 758.67 g, respectively, while decreasing the head weight of the Bohooth genotype under control conditions to 415.67 g. Moreover, the concentration of 12 ppm of ABA with the Bohooth genotype outperformed and yielded the highest 1000-grain weight, 36.00 g. In contrast, the control treatment with the Warkaa genotype recorded the lowest average, reaching 27.00 g. ABA plays an effective role in enhancing grain filling features, accelerating starch build-up by raising related enzyme activities, and regulating levels of grain endogenous hormones, consequently contributing to adequate grain filling (Yu et al., 2024).

The spraying of 12 ppm ABA significantly affected the grain yield in Al-Khair and Giza genotypes, with maximum yields of 8.11 and 8.12 t ha^{-1}, respectively, when compared to the control treatment and the Bohooth genotype, which had the lowest yield, reaching 4.77 t ha^{-1}. Exogenous spraying of 12 ppm ABA for the Giza genotype positively and significantly increased biological yield by an average of 18.19 t ha^{-1}, over the control treatment with the Warkaa genotype, which had the lowest 14.15 t ha^{-1}. In addition, the harvest index was significantly affected by the interaction of exogenous ABA spraying and sorghum genotypes. The interaction between 12 ppm ABA spraying and the Khair genotype significantly registered a maximum harvest index of 47.05%, while the control treatment with the Bohooth genotype registered a minimum of 29.15%. The above results agree with Al-Jawaheri and Alogaidi (2020), who observed a beneficial interaction between ABA concentrations and rice varieties in grain panicle^{-1}, weight of 1000 grains and total grain yield. Yu et al. (2024) found that maize hybrids reveal a significant increase in weight of 1000 grains and grain yield after following ABA treatment over the control. Naqqash et al. (2024) obtained a positive and significant interaction with exogenous spraying of ABA on canola (*Brassica napus* L.) cultivars, which resulted in an increased number of seeds silique^{-1}, weight of 1000 grains, seed yield and biological yield as compared to the control treatment.

Table 6
Interaction of abscisic acid (ABA) with sorghum genotypes on the studied indices

ABA (ppm) × Genotypes	Plant Height (cm)	Plant Leaves (Leaf Plant^{-1})	Leaves Chlorophyll Content (mg g^{-1} Fresh Weight)	Plant Leaf Area (cm^2)	Grains Head^{-1}	Head Weight (g)	1000-grain Weight (g)	Grain Yield (t h^{-1})	Biological Yield (t ha^{-1})	Harvest Index (%)
0× Bohooth	169.40 ab	10.20 bcd	43.78 c	5578.41 ef	2360.00 abc	415.67 e	27.67 f	4.77 d	14.97 fg	29.15 f
0×Giza	145.00 fg	9.93 bcd	42.15 cd	5934.47 b	2337.33 abc	504.00 d	27.33 f	5.86 bc	15.87 def	37.28 cde
0×Al-Khair	148.18 efg	10.40 bc	48.64 ab	5751.35 cd	2346.00 abc	548.67 cd	29.00 ef	5.10 cd	15.17 fg	33.56 c-f
0×Warkaa	141.69 g	9.10 d	40.31 d	5468.55 g	2279.00 c	476.00 d	27.00 f	5.67 cd	14.15 g	40.07 abc
9×Bohooth	172.53 a	11.67 a	43.59 c	5685.56 cde	2432.00 abc	540.67 cd	30.00 cde	5.19 cd	17.00 a-d	30.53 ef
9× Giza	149.67 d-g	10.40 bc	42.39 cd	6010.21 b	2440.00 abc	574.00 c	28.67 ef	6.72 b	17.54 ab	38.70 bcd
9× Al-Khair	157.58 cd	10.80 ab	49.15 ab	5739.27 cd	2405.00 abc	571.67 c	31.00 bcd	6.66 b	16.71 b-e	39.92 abc
9× Warkaa	152.30 def	9.27 cd	42.22 cd	5568.07 fg	2304.67 bc	546.00 cd	29.00 def	5.59 cd	15.52 ef	35.97 c-f
12×Bohooth	176.00 a	11.13 ab	44.92 bc	5787.32 c	2500.67 ab	726.33 a	36.00 a	5.12 cd	17.61 ab	31.85 def
12× Giza	152.47 def	10.90 ab	45.65 bc	6141.52 a	2535.33 a	758.67 a	33.00 b	8.12 a	18.19 a	44.91 ab
12× Al-Khair	161.72 bc	11.10 ab	51.51 a	5934.02 b	2414.67 abc	762.67 a	32.33 b	8.11 a	17.26 abc	47.05 a
12× Warkaa	155.80 cde	9.53 cd	42.61 cd	5659.27 def	2440.00 abc	668.00 b	32.00 bc	5.85 bc	16.15 c-f	36.30 c-f

Note. In the same column, similar letters do not differ significantly

CONCLUSION

The obtained results confirm that the positive effects of ABA, particularly 12 ppm concentration under high temperature conditions, meaningfully improved the capability of the sorghum plants to tolerate the bad effects of abiotic stress and improved their growth and yield. So, it can be concluded that the foliar spraying of abscisic acid significantly improves sorghum growth performance and yield attributes, effectively mitigates abiotic stress responses, which leads to better growth and productivity compared with not spraying. Moreover, foliar spray of exogenous abscisic acid exhibited differences due to the different sorghum genotypes. Increasing the sorghum genotype growth and yield is mainly related to the enhancement of plant leaves, leaf area, leaf chlorophyll content and then photosynthesis in leaves. The sorghum genotypes exhibited highly significant performance across all studied indices; the findings indicated that the impact of exogenous ABA on the plants mainly depended on the sorghum genotypes. Both genotypes, Khair and Giza, significantly outperformed in most of the studied indices, while the binary interaction of 12 ppm ABA with the Giza genotype recorded the highest grain yield. Therefore, it can be considered the best treatment proposed in this study. Future studies should explore the impacts of these treatments across different crops to promote the agricultural sustainability of semi-arid regions.

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Diversity and Ecological Insights of Fruit Bats in Durian Orchards of Sarawak, Malaysian Borneo

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ABSTRACT

Pteropodid bats are important pollinators for tropical fruits like durian (*Durio zibethinus*), which is very important for both the economy and culture of Southeast Asia. This study aimed to examine the ecological interactions and habitat relationships of pteropodid bats in durian orchards. From August 2021 to November 2022, mist-netting surveys were done at 13 orchards and documented 839 individuals from ten pteropodid species. *Cynopterus brachyotis* was the most common species seen. Floral observations showed that anthesis in *D. zibethinus* started at roughly 16:30 with the opening of flower buds, succeeded by anther dehiscence at 19:00, coinciding with the initiation of feeding activity in pteropodid bats. Pollen analysis verified floral visitation, revealing that 82% of pollen-positive captures were attributed to *C. brachyotis*. Bat captures peaked during the flowering period, especially from September to November. Principal Component Analysis (PCA) and Generalised Linear Mixed Models (GLMMs) were used to look at the habitat, and they showed that the size of the durian orchard (ha) was the sole important factor that affected the number of bats. Other variables, such as orchard age and the quantity of flowering trees, did not exhibit statistical significance. These results show how important durian orchard sites are for bat-mediated pollination services and propose that conservation should be a part of how orchards are managed.

Keywords: Habitat association, mist netting, pollination, pteropodidae

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INTRODUCTION

Old World fruit bats, or pteropodid bats, are necessary for the pollination of several plants, such as durian (*Durio zibethinus*), petai (*Parkia speciosa*), mango (*Mangifera indica*), and kapok (*Ceiba pentandra*) (Aziz et al., 2021; Ramírez-Fráncel et al., 2022; Stewart & Dudash, 2018). In countries like Thailand, Malaysia, Indonesia, Brunei, Vietnam, Myanmar, Cambodia, and Laos, durian is very important to both the economy and culture (Drenth & Guest, 2004). From 2015 to 2017, the world produced almost 2.3 million metric tonnes of durian, which shows how important it is as a staple fruit in Southeast Asia (Altendorf, 2018). Pteropodid bats are the main pollinators in this business, and the orchards are where these bats get their food and shelter (Aziz et al., 2021). Studies in Indonesia showed that pollen analysis can effectively identify the pattern of floral visitation and the functional groupings among the fruit and nectar bats (Bumbut et al., 2016; Maryati et al., 2008; Soegiharto et al., 2010). Different bat species can be better or worse at pollinating, and *Eonycteris spelaea* is known for being quite good at it since it carries a lot of pollen and has particular ways of finding food (Low et al., 2021; Mohammad-Shah et al., 2021). While the bats eat, they move the pollen around. Although *Durio zibethinus* is capable of limited self-pollination, the bat's actions helped the cross-pollination and substantially enhanced genetic diversity, fruit set, fruit size, and overall yield quality (Baqi et al., 2022; Honsho et al., 2007). Habitat loss, poaching, and other human-caused problems are major risks to many bat species, which could hurt natural pollination services (Aziz et al., 2016; Meyer et al., 2016; Mohd-Azlan et al., 2022). The Sarawak Wild Life Protection Ordinance 1998 (WLPO 1998) protects all chiropteran species in the State of Sarawak because of these risks. Anyone who hunts, kills, captures, sells, or says they will sell any species of fruit bat might be fined RM10,000 and sent to jail for up to a year. These species are protected in Sarawak, but not much is known about where they live and how they interact with modified landscapes like durian orchards.

Identifying bat species that inhabit durian orchards can aid the prioritisation of conservation efforts, including the protection of roosting areas, mitigation of disturbances, and development of bat-friendly habitats (Meli et al., 2024). These efforts not only protect bat populations, but they also help keep the ecosystem next to them in balance and full of all kinds of plants and animals. Conserving nearby forests, using fewer pesticides when bats are foraging, and planting more flowering plants to feed bats are all ways to get pollinators to visit all the time (Muchhala & Jarrin, 2002; Oliveira et al., 2020; Ripperger et al., 2015). Studies showed that by having a healthy bat population, durian harvests and quality increase, which benefits farmers, consumers, and local economies (Baqi et al., 2022; Muhammad et al., 2021).

Considering the interdependent functions of bats, ecosystems, and agriculture, the presence of pteropodid bats in durian orchards is both ecologically significant and practically essential (Muchhala & Jarrin, 2002; Meli et al., 2024). Protecting these essential

pollinators and their ecosystems would ensure the sustainability of an industry that sustains millions of livelihoods while enhancing biodiversity and ecological well-being (Altendorf, 2018; Drenth & Guest, 2004; Kasso & Balakrishnan, 2013).

Ongoing research on bats in Borneo mainly focuses on the diversity, genetics, ecology and conservation (e.g. Habeebur-Rahman et al., 2025; Lok et al., 2021; Mc Arthur et al., 2024; Morni et al., 2025; Yoh et al., 2023). While most of this research focuses on protected and forested areas, very little attention is given to bats in human-modified landscapes. Studies on bats in plantations such as oil palm and in Sarawak have shown that fruit bats dominate the species diversity and abundance. However, no research on bats has been conducted in fruit orchards such as durian, an ecologically important crop for some pteropodids. in Borneo. Therefore, this research is the first in Borneo to (1) document the species of pteropodid bats visiting durian orchards, (2) investigate the floral traits of *D. zibethinus* and its interaction with pteropodid bats, and (3) investigate the influence of habitat heterogeneity on the presence of pteropodid bats in the durian orchards.

MATERIALS AND METHODS

Study Site

This study was conducted in 13 durian orchards located at various sites across Sarawak between August 2021 and November 2022 (Figure 1). At each site, ten mist nets were set for three consecutive nights. The placement of the nets was between tree rows and near flowering durian trees, targeting the bats foraging within the orchard. All mist nets were checked regularly at 15-30 minute intervals after dusk until 2300 hours and resumed the following mornings from 0530 hours. The species identification referred to Phillipps and Phillipps (2018). All bat individuals were released upon morphological measurements and species identification.

Bat Abundance and Conservation Status

For each bat species, the analysis presented the mean number of individuals per night, percentage of species composition, and number of individuals observed through all sampling nights, along with their IUCN conservation status (IUCN, 2025).

Floral Observation and Pollen Analysis

Observations of *D. zibethinus* were conducted at a mixed-fruit orchard in Serian (1°11'54.6"N 110°32'51.2"E, 11 m elevation). To record the process of flower-opening, flowers undergoing anthesis were photographed with a digital camera at two-hour intervals for over 24 hours. The progress of the floral development (from budding to wilting) was categorised into percentage (%) for every two hours. The mean percentage of blooming was plotted to observe the trend.

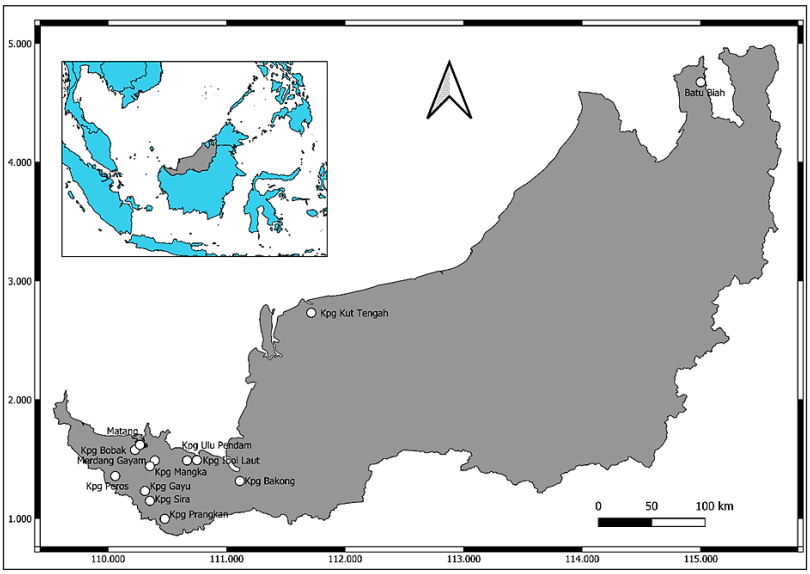

Figure 1. Sampling distribution of pteropodid bats in Sarawak from August 2021 to November 2022

Pollen grains were collected from pteropodid bats using a hand-picking technique (Low et al., 2021). Pollen adhered to the bats' bodies was carefully removed and transferred into Eppendorf tubes containing FAA (Formalin-Acetic Acid-Alcohol) solution to preserve the samples (Thompson, 2016). The tubes were immediately stored in a polystyrene box with ice packs to prevent drying. After being brought to the lab, the pollen samples were made ready for identification (Baqi et al. 2022). Using a micropipette, pollen grains were moved on glass slides so that they could be seen under a microscope. A Leica DM500 compound microscope with a Leica ICC50 W camera (10X magnification) was used to identify the object. Pollen was identified using comparisons with recognised pollen types collected from the study region.

Habitat Characteristics

The main types of landscapes around the study sites were (1) rubber plantations, (2) oil-palm plantations, (3) mixed-fruit orchards, and (4) places where people live. We recorded the following parameters for each site: (1) the surrounding matrices (n) (forest/oil palm plantation/rubber plantation), (2) the age of the orchard (n), (3) the area of the orchard (hectares), (4) the number of flowering durian trees (n), (5) the number of other flowering trees (n), (6) the total number of durian trees (n), and (7) the distance of the orchards to the forest (m) (Appendix 1). We got this information by measuring things directly in the field, looking at them, and talking to orchard owners. GPS devices were utilised to find the locations of orchards and the distance to the nearest forest edge. Tree counts and species identification were done on site.

This study used Principal Component Analysis (PCA) to find the variables that had the largest effect on the results. PCA simplifies intricate datasets by pinpointing the most significant underlying elements or components that organise species assemblages and environmental traits, hence enhancing data visualisation and interpretation. Utilising the principal components derived from PCA, a Generalised Linear Mixed Model (GLMM) with a Poisson distribution was employed to analyse the impact of habitat heterogeneity factors on the overall abundance of pteropodid bats in durian orchards. We tried 16 different models to cover all the conceivable combinations of the four habitat heterogeneity variables. All analyses were conducted using RStudio (version 2024.9.1.394), with packages such as "readxl", "corrplot", "ggfortify", "gridExtra", "carData", "car", "factoextra", "FactoMineR", "labelling", "farver", "pscl", "boot", "ggplot2", "lme4", "MuMIn", "dplyr" and "performance".

RESULTS

Species Abundance and Composition

A total of 839 individuals of pteropodid bats representing ten species were recorded across 13 durian orchards in Sarawak. This species accounts for approximately 56% of the total pteropodid bat species recorded in Borneo. Among these, three species were nectarivorous: *Eonycteris spelaea*, *Eonycteris major*, and *Macroglossus minimus*, while the remaining seven species were frugivorous, including *Balionycteris maculata*, *Cynopterus brachyotis*, *Cynopterus horsfieldii*, *Cynopterus minutus*, *Dyacopterus spadiceus*, *Penthetor lucasi*, and *Rousettus amplexicaudatus* (Table 1, Figure 2).

Table 1
The checklist of the bat species in durian orchards, Sarawak, Malaysia

Species	Mean ±S.D. (indv./night)	Species Composition(%)	Diet	IUCN 2025
Balionycteris maculata	0.05±0.23	<1	Frugivorous	LC
Cynopterus brachyotis	12.95±3.60	60	Frugivorous	LC
Cynopterus horsfieldii	0.18±0.42	1	Frugivorous	LC
Cynopterus minutus	0.90±0.95	4	Frugivorous	LC
Dyacopterus spadiceus	0.38±0.62	2	Frugivorous	NT
Eonycteris major	0.05±0.23	<1	Nectarivorous	NT
Eonycteris spelaea	2.87±1.69	14	Nectarivorous	LC
Macroglossus minimus	2.13±1.46	10	Nectarivorous	LC
Penthetor lucasi	1.92±1.39	9	Frugivorous	LC
Rousettus amplexicaudatus	0.03±0.16	<1	Frugivorous	LC

Note. *Individual (Indv.); Least Concern (LC); Near Threatened (NT)

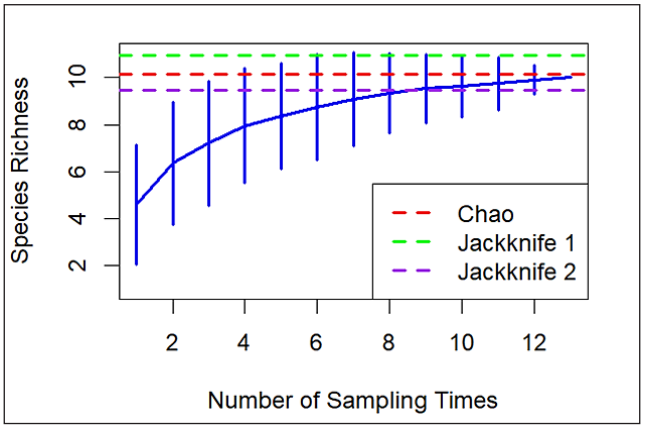

Figure 2. Species accumulation curve of pteropodid bats captured in durian orchards against the number of samplings conducted in Sarawak from August 2021 to November 2022

Anthetic Progression

Observations of *D. zibethinus* floral development revealed that from 1100 hours to 1600 hours, individual buds within each cluster gradually enlarge as they mature. The bracts and sepals begin to loosen, indicating the approach of anthesis. Between 1600 and 1900 hours, petals started to unfold, progressively exposing stamens and pistils as the flower entered full bloom. By approximately 2100 hours, flowers were fully open and emitted a distinctive scent, which is a critical attractant for nocturnal pollinators such as bats and insects. During this phase, the anthers release pollen, facilitating pollination when it contacts the receptive stigma of another flower, consistent with previous studies (Honsho et al., 2004; Thorogood et al., 2022). By 0300 hours the following day, the flowers begin to wilt, and unpollinated flowers abscise and fall from the tree within a day or two, completing their life cycle (Figure 3).

Approximately 26% of the flower buds within a cluster show partial opening by 1100 hours. This proportion increased steadily, with 40-50% opening by early evening. By 1900 hours, approximately 95% of flowers were fully or nearly full bloom. After 0100 hours, the flowers started to show signs of wilting before eventually dropping.

Pollen Analysis

From the ten pteropodid species recorded during sampling, only four species were observed carrying pollen, from which a total of 68 pollen samples were collected. The remaining six species were rarely captured and did not carry detectable pollen. *C. brachyotis* had the highest number of pollen samples (n = 56, 82%), followed by *E. spelaea* (n = 6), *P. lucasi* (n = 5) and *M. minimus* (n = 1). The highest pollen sample count was recorded in August, suggesting this month as the peak flowering season in Sarawak (Figure 4).

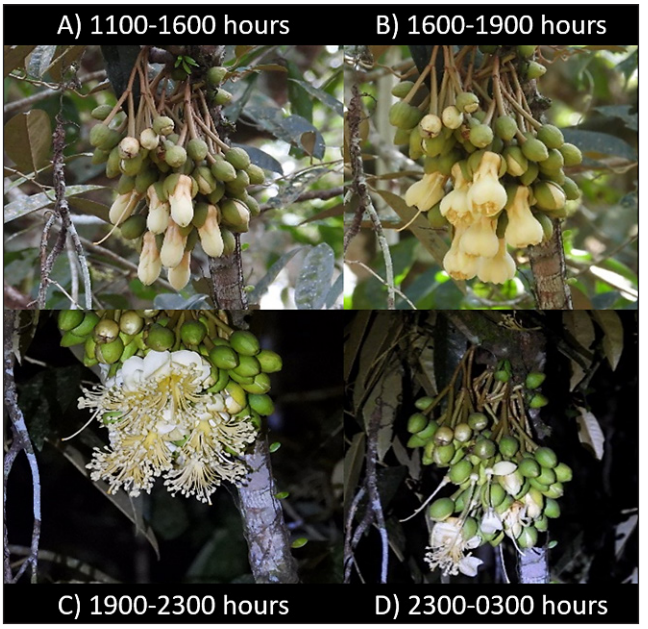

Figure 3. Floral development of *D. zibethinus* from bud initiation to flower abscission with a corresponding timeline

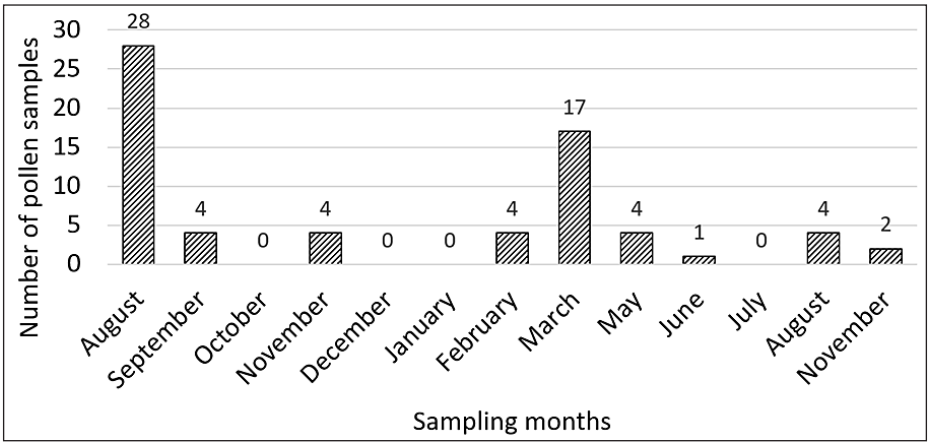

Figure 4. The number of pollen samples collected from August 2021 to November 2022

A linear regression analysis demonstrated a statistically significant positive relationship between the percentage of the flowering progress and the number of pteropodid bats captured, indicating that increased flowering activity is associated with higher bat visitation (Linear regression; $F = 17.41$, $P = 0.02$, $R^2 = 0.61$).

Effect of Habitat Heterogeneity on Bat Species Abundance

Principal component analysis (PCA) was performed to identify the major contributing variables related to habitat heterogeneity. Three principal components accounted for 86% of the total variation. The distance of the orchard to the forest, the number of durian trees and the number of flowering durian trees were negatively correlated with orchard age. Additionally, the orchard area and surrounding matrices showed a negative correlation with the other flowering trees. Based on the PCA results, the variables retained for further analysis were the number of durian trees, other flowering trees, orchard age, and orchard area, as these variables contributed most to the variation in the data and showed strong associations with the principal components that explained the majority of the variance. Specifically, the number of durian trees showed a high loading on PC1 (−0.463), while orchard age (−0.430) and orchard area (−0.420) loaded strongly on PC2. The number of other flowering trees also contributed notably to PC2 (0.341) (Table 2).

A total of 16 Generalised Linear Mixed Models (GLMMs) were run to assess the influence of habitat heterogeneity factors, comprising orchard area (ha), orchard age, number of durian trees, and number of other flowering trees on the abundance of pteropodid bats in durian orchards. The five best-fitting models based on AICc values are summarised in Table 3.

Table 2
Component matrix derived from the result of PCA

Factors	Component Matrix		
	PC1	PC2	PC3
Number of matrices	-0.289	-0.528	-0.272
Area of the orchard (hectare)	-0.347	-0.420	0.388
Age of the orchard	0.137	-0.430	0.623
Number of flowering durian trees	-0.418	0.382	0.370
Number of other flowering trees	0.332	0.341	0.469
Number of durian trees	-0.463	0.296	-0.099
Distance of the orchards to the forest	-0.528	0.107	0.143

Table 3
Results of the GLMM model examine the effect of surrounding habitat heterogeneity factors on the abundance of pteropodid bats in durian orchards

Model	Included Variables	AICc	ΔAiCc	Weight	p-value
2	Area of the orchard	130.42	0	0.46	0.002*
10	Area of the orchard + Number of durian trees	132.54	2.12	0.16	0.999
6	Area of the orchard + Other flowering trees	133.04	2.62	0.12	0.351
5	Other flowering tree	133.77	3.35	0.09	0.050
4	Area of the orchard + age of the orchard	133.91	3.49	0.08	0.999

Note. *Significant value

The best-performing model (Model 2, AICc = 130.42) included only orchard area as a fixed effect and revealed a significant positive association between orchard area and the abundance of pteropodid bats (Weight = 0.46, p = 0.002). Other predictors, including orchard age, number of durian trees, and number of other flowering trees, were not statistically significant in any of the top models. Additionally, when the linear predictor for the species-level interaction with orchard area was tested, no significant relationship was found for any of the species (P>0.05; Intercept Estimate: 1.98-5.18).

DISCUSSIONS

Species Composition and Ecological Roles

The current study is the first to report the distribution of pteropids in durian orchards in Sarawak, Malaysia, Borneo. In the study area, *C. brachyotis* was the most abundant species recorded (n = 505, 60%), suggesting that they are adaptable to orchard ecosystems and utilise this habitat for foraging (Campbell et al., 2006; Jayaraj et al., 2012; Karuppudurai & Sripathi, 2018). This is followed by the nectivorous, *E. spelaea* (n=112, 13%) and *M. minimus* (n=83, 10%). The fact that these species are essential pollinators, in particular for durian and other flowering plants near the habitat, suggests that the orchards not only supply sources of food but also play a role in maintaining the ecological niche by providing support for populations of pollinators (Demestihas et al., 2017; Garratt et al., 2023).

This study also recorded a singleton individual of *R. amplexicaudatus.* This species is a high-flying species, therefore the standard mist-netting method is less likely to trap this species. *Rousettus amplexicaudatus* is commonly recorded in krast regions as compared to human-altered areas. Previous studies have documented numerous individuals in orchards (e.g. Heideman & Utzurrum, 2003; Pounsin et al., 2016; Tanalgo et al., 2021). Our study also documented two IUCN RedList Near Threatened species, *D. spadiceus* and *E. major*. This suggests that the durian orchard may function as a sanctuary for these bats, especially in the areas where their populations are fragile due to habitat loss.

Flowering Ecology and Bat Pollination

This study contributed to the ongoing work of looking into the durian flowering ecology in Southeast Asia (Bumrungsri et al., 2009; Honsho et al., 2004; Masri, 1999; Ng et al., 2020). The blooming season in Sarawak is different in different parts of the state. It started in the northern part, moving south and then west across the state. This is influenced by the weather changes such as the temperature, rainfall and soil conditions, affecting the flowering patterns (Rajandran et al., 2024; Suwanseree & Yapwattanaphun, 2017). These alter the fruit's availability of fruit, the harvest time and farmers' profits.

Durio zibethinus's flower started to bloom in the evening and stayed open all night. This coincides with the activity pattern of the pteropodid bats, highlighting the species adaptation to bat-mediated pollination. Previous studies have demonstrated the synergy of the durian-bat pollination (Bumrungsri et al., 2013; Sheherazade et al., 2019; Low et al., 2021).

The detection of pollens on pteropodid bats, mostly on the heads, faces and fur of the bats, showed their important function as pollinators for *D. zibethinus*. These showed that they do effectively interact with the flower reproductive systems while eating, highlighting the significance of head and fur contact in improving pollen transfer efficiency (Ng et al., 2020; Pontes et al., 2024; Sheherazade et al., 2019).

The varying amounts of pollen among species may result from differences in their feeding habits. *Eonycteris spelaea* and *M. minimus* are two examples of nectarivorous species with long tongues that help them get nectar (Phillips & Phillips, 2018). This could help them eat pollen or keep pollen from being externally retained on their fur, which is not the case with other frugivorous species that eat a wide range of foods (Bumrungsri et al., 2013; Stewart & Dudash, 2018). *Cynopterus brachyotis*, which is commonly thought of as a generalist feeder, actually helps with pollination by visiting flowers often. Therefore, pollen should be seen as proof that flowers were visited, not as a way to see how well pollination worked. Similar pollen studies in Indonesia have demonstrated species-specific floral associations and functional grouping among fruit and nectar bats (Maryati et al., 2008; Soegiharto et al., 2010). While certain insects visit durian blossoms, their effectiveness in pollination is inferior to that of pteropodid bats (Fleming et al., 2009).

The cross-pollination has been consistently shown to enhance fruit development and marketable yield (Honsho, 2007). In this context, pollinator presence, particularly by mobile vertebrate pollinators such as pteropodid bats, plays a functionally essential role in sustaining the optimal yield and durian's fruit quality (Cervancia, 2018). The ecological reliance on cross-pollination reinforces the importance of maintaining bat populations within agricultural landscapes.

Influence of Habitat Heterogeneity

Among the environmental parameters evaluated, only the size of the orchard showed a statistically significant link to the number of pteropodid bats. The positive link between the size of an orchard and the number of bats suggests that connected orchards may provide better foraging possibilities and roosting sites for pteropodid bats. Larger orchards may have more nectar and pollen because they have more flowers, which is in line with research that shows that larger habitats have more bats because there is less competition and better access to resources (Mohd Nokin & Ramli, 2022).

The orchard size may also correlate with the ecological characteristics of pteropodid bats that have large home ranges and can fly around a lot, helping them to keep track of available resources in different places. As an example, Acharya et al. (2015) reported that *E. spelaea* has large home ranges and changes its foraging pattern depending on how many flowers are available (Acharya et al., 2015). During the peak flowering season of the durian flower, the bats do not need to rely much on other flowering plants in the area.

Previous studies have reported that habitat heterogeneity enhances bat activity and diversity by offering multiple resources and microhabitats (Frey-Ehrenbold et al., 2013). Despite this, the impacts of habitat heterogeneity are scale-dependent, differing between local and landscape levels (Mendes et al., 2017). Landscape composition and connectivity are known to influence bat presence and activity (Hale et al., 2012), suggesting that broader spatial factors may be more significant than within-orchard variation for highly mobile fruit bats.

Aziz et al. (2021) have emphasised the essential ecological function of pteropodid bats in pollination and seed dissemination services in both natural and agricultural settings. While most studies have focused on forest landscapes, the study of pteropodid bats in fragmented agricultural settings is still understudied (Ferreira, 2015; Meyer et al., 2016; Struebig et al., 2008). This study highlighted the significance of the orchard area, which supports at least 56% of the Pteropodid bat species identified in Sarawak.

Conservation Strategies

The findings from this study have practical implications for durian orchard management and bat conservation. Since orchard area significantly influences bat species richness and abundance, maintaining larger contiguous orchard plots or integrating smaller orchards into a connected landscape could enhance foraging opportunities. Conservation methods on a landscape scale can make it easier for orchards and natural ecosystems to link (Kay et al., 2018). Setting up habitat corridors can make it easier for bats to migrate around and mix their genes. Adding orchards to larger regional conservation plans can also make them more valuable to the environment (Cleary et al., 2017; Razgour et al., 2014; Ripperger et al., 2014).

The next strategy is to take care of the orchard's ecosystem. Providing native flowering plants and trees such as banana (*Musa acuminata*), domestic jackfruit (*Artocarpus integer*), and rambutan (*Nephelium lappaceum*) in the area will provide a good roosting and foraging site for the bats (Corlett, 2005; Lim et al., 2018).

The other strategy is to reduce pesticide usage. The pesticides can drastically affect the survivability of the bats (Oliveira et al., 2020; Torquetti et al., 2021). The Integrated Pest Management (IPM) needs to be adopted to ensure the negative effects of the pesticides on the bats and their food source can be reduced (Liu et al., 2024). Besides, raising awareness among farmers and local communities in the area on the bat's role in ecology

and economic aspects is important in order to shift the negative perception towards bats and foster coexistence (Aziz et al., 2021; Ejotre et al., 2022).

While all bats are protected in Sarawak according to SWLPO (1998), their presence in fruit orchards is sometimes regarded as a pest by local farmers, who are allowed to protect their crops from potential pest species under section 42A of the same Ordinance. Therefore, fruit growers and farmers should be made aware of the role of fruit bats as pollinators for durians, which in turn may increase the yield (Harahap & Yonariza, 2022; Sheherazade et al., 2019). Lastly, it is also important to reduce the hunting pressures and human disturbances at bat roosting locations to make sure the species survives (Frick et al., 2020; Morni et al., 2018).

All of these conservation strategies can be implemented to encourage environmentally friendly farming. The orchard owners can protect the pollinator populations, keep the ecosystem in balance, and make sure more consistent durian production over time.

CONCLUSION

This study showed that the durian orchards are not only beneficial for the economy, but also can serve as an important habitat for a wide range of bat species, including important pollinators, seed dispersers and species of conservation interest. The ecological relationship between the bats and the durian orchard is mutually beneficial, and their protection, therefore, should be prioritised in future conservation planning.

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SUPPLEMENTARY DATA

Table S1
List of habitat heterogeneity factors of 13 durian orchards in Sarawak (Acronyms for sites are given below the table)

Factors	Sites												
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S12**	**S13**
Number of surrounding matrices (n)	2	1	2	1	1	1	1	2	1	1	1	2	1
Age of the orchard (years)	53	86	57	40	66	48	44	50	15	33	30	27	32
Area of the orchard (hectare)	3.22	0.79	1	0.7	1.12	0.75	1	1.26	0.72	0.26	0.94	0.59	0.32
Number of durian trees (n)	10	7	10	5	5	7	5	10	20	5	5	10	7
Number of flowering durian trees (n)	4	3	0	0	1	2	1	1	7	1	1	1	0
Number of other flowering trees (n)	2	3	3	3	4	3	3	2	3	4	3	2	2
Distance of the orchard to the nearest forest (m)	345	83	79	66	60	147	84	162	310	75	84	83	148

Note. S1, Kampung Ulu; S2, Kampung Peros; S3, Kampung Sira; S4, Kampung Bobak; S5, Kampung Gayu; S6, Merdang Gayam; S7, Kampung Prangkan; S8, Kampung Iboi Laut; S9, Matang; S10, Kampung Mangka; S11, Kampung Kut Tengah; S12, Kampung Bakong; S13, Batu Biah

Table S2
Number of pteropodid bat species captured from 13 sampling sites in durian orchards between August 2021 and November 2022 in Sarawak (Acronyms for sites are given below the table)

Species	Sites													Relative abundance	IUCN 2025
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13		
Balionycteris maculata	0	0	0	1	0	0	1	0	0	0	0	0	0	0.24	LC
Cynopterus brachyotis	218	16	17	12	1	46	22	82	30	26	10	2	23	60	LC
Cynopterus horsfieldi	0	0	0	0	0	6	0	0	0	0	1	0	0	0.83	LC
Cynopterus minutus	0	0	2	0	0	0	5	10	9	3	3	2	1	4.17	LC
Dyacopterus spadiceus	1	0	0	5	0	3	0	0	0	0	0	4	2	1.79	NT
Eonycteris major	0	0	1	0	0	0	1	0	0	0	0	0	0	0.24	LC
Eonycteris spelaea	0	2	16	0	7	0	11	0	13	34	0	27	2	13.35	LC
Macroglossus minimus	1	2	1	0	1	9	1	46	0	0	11	3	8	9.77	LC
Penthetor lucasi	0	11	30	4	12	7	0	0	2	0	0	9	0	8.94	LC
Rousettus amplexicaudatus	0	0	0	0	0	0	1	0	0	0	0	0	0	0.12	LC
Number of Individuals	220	31	67	22	21	71	42	138	54	63	25	47	36		
Number of species	3	4	6	4	4	5	7	3	4	3	4	6	5		
Shannon-Wiener Index	0.058	1.06	1.28	1.12	0.98	1.11	1.30	0.87	1.09	0.84	1.11	1.29	1.041		
Simpson Index	0.018	0.60	0.68	0.62	0.56	0.55	0.64	0.53	0.60	0.54	0.63	0.62	0.54		
Species evenness	0.35	0.72	0.60	0.76	0.66	0.61	0.52	0.79	0.74	0.77	0.76	0.60	0.57		

Note. S1, Kampung Ulu; S2, Kampung Peros; S3, Kampung Sira; S4, Kampung Bobak; S5, Kampung Gayu; S6, Merdang Gayam; S7, Kampung Prangkan; S8, Kampung Iboi Laut; S9, Matang; S10, Kampung Mangka; S11, Kampung Kut Tengah; S12, Kampung Bakong; S13, Batu Biah

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Phylogeography and Genetic Structure of the Giant Freshwater Prawn (Decapoda: Palaemonidae) in Peninsular Malaysia using Mitochondrial DNA Analysis

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ABSTRACT

The Giant Freshwater Prawn (GFP), *Macrobrachium rosenbergii,* is a cornerstone of global freshwater aquaculture, especially in Southeast Asia, due to its increasing demand for high-quality seed stock and market value, yet the genetic structure of its natural populations in Malaysia remains poorly understood. This study evaluated the population structure and genetic diversity in the wild GFP across Peninsular Malaysia to identify superior strains for sustainable breeding programmes. This study analysed a 572 base pair segment of the mitochondrial cytochrome *c* oxidase 1 (COX1) gene from 149 GFP individuals collected from six river systems across Peninsular Malaysia. Twelve distinct haplotypes were identified, with phylogenetic analysis showing polyphyletic relationships among populations. The Kelantan river population exhibited the highest level of genetic diversity (π = 0.0024) and haplotype diversity (h = 0.5885), whereas the Perak river population showed no polymorphism. These results suggest a historical population bottleneck that drastically reduces the effective population size, in turn leading to loss of rare alleles and thus the increase in genetic homogeneity. As indicated by negative Tajima's D and Fu's FS values, significant deviations from neutrality, together with unimodal mismatch distributions,

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suggest a historical bottleneck followed by rapid population growth and the subsequent accumulation of rare mutations. These findings provide a critical genetic baseline for the management of wild stocks and the selection of high-diversity founder populations for the GFP aquaculture industry in Malaysia.

Keywords: COX1 gene, genetic diversity, genetic variation, *Macrobrachium rosenbergii,* population structure

INTRODUCTION

The Giant Freshwater Prawn (GFP), locally known as "Udang Galah" in Malaysia or scientifically known as *Macrobrachium rosenbergii*, is a highly sought-after, commercially valuable species globally (Yu et al., 2019). In 2022, global production reached 294 thousand tons, making it one of the most extensively cultured decapod species, and its production is essential to the aquaculture industries globally (Zheng et al., 2024). The prawns are distributed from Northwest India down to Southeast Asia, Papua New Guinea, the Philippines, and Northern Australia (Banu & Christianus, 2016). The GFP has four distinct stages in its life cycle, which are the egg, larva, juvenile and adult. While mating occurs throughout the year, peak breeding periods are observed during the monsoon seasons (October to January, and March to May), when increased rainfall and optimal water temperatures trigger downstream migration and mass spawning (Mitra et al., 2005). Notably, the successful development of GFP larvae requires brackish water, as survival rates are drastically reduced if they are not transferred to such an environment within a few days of hatching. In their natural habitat, GFP larvae primarily feed on zooplankton, small insects and the larvae of other aquatic invertebrates. After mating, gravid females migrate towards brackish water where the eggs hatch as free-swimming larvae before transitioning to post-larvae. These post-larvae then migrate upstream into freshwater systems, where they continue their life cycle into adulthood, a critical aspect of their survival and reproductive success. Moreover, this migration also serves to reduce predation risk for the prawns (Li et al., 2010).

Understanding the biodiversity and population structure in aquatic ecosystems requires genetic analysis of aquatic species. The Cytochrome c oxidase subunit 1 (COX1) gene is a mitochondrial marker widely adopted in DNA barcoding for species identification and classification (Hebert et al., 2003). The COX1 gene has proven particularly effective in distinguishing closely related species, identifying cryptic taxa and investigating intraspecific diversity (Trivedi et al., 2016). This gene has been successfully utilised in a variety of organisms, including both vertebrates and invertebrates (Oba et al., 2015; Tavares & Baker, 2008; Trivedi et al., 2016), yet vice versa for plants and some species of fungi (Ward et al., 2005). Compared to other mitochondrial genes, COX1 gave the signal for a better

phylogenetic analysis (Strüder-Kypke & Lynn, 2010). Even though still in the preliminary level, application of COX1 already showed positive results for barcode dinoflagellates, *Paramecium* sp., and *Nemertea* (Stern et al., 2010; Sundberg et al., 2016; Zhao et al., 2013). So, mixed analyses of nuclear and mitochondrial markers might assist in narrowing the knowledge gap (Blaxter, 2016). In this study, we aim to analyse the genetic structure of the wild GFP populations in Peninsular Malaysia using the mitochondrial cytochrome c oxidase 1 (COX1) gene. This approach enables the assessment of genetic diversity and population differentiation, which is important for identifying genetically diverse and potentially superior strains for aquaculture. The findings can support the selection of suitable broodstock, improve selective breeding strategies, reduce the risk of inbreeding, and contribute to sustainable aquaculture management and conservation of valuable wild genetic resources.

MATERIALS AND METHODS

Sampling Locations

The GFP samples for this study were collected from various rivers across Peninsular Malaysia (Table 1), including Sungai Linggi (Negeri Sembilan), Sungai Perak (Perak), Sungai Pahang (Pahang), Kuala Selangor (Selangor), Sungai Selehong (Kelantan), and Sungai Kerian (Pulau Pinang). These samples were obtained from wild populations and subsequently stored at -20 ˚C in the Genetics Laboratory of Universiti Putra Malaysia prior to DNA extraction.

Table 1
Sample collection from six different populations in Peninsular Malaysia

Location	Sample Size
Sungai Linggi, Negeri Sembilan	20
Sungai Perak, Perak	29
Sungai Pahang, Pahang	23
Kuala Selangor, Selangor	24
Sungai Selehong, Kelantan	30
Sungai Kerian, Pulau Pinang	23

DNA Extraction and Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from the muscle tissue of GFP samples using the ReliaPrep gDNA Tissue Miniprep System (Promega Corp, Madison, USA) according to the manufacturer's instructions. The extracted DNA was kept at 4 °C prior to the PCR protocol. Approximately 25 mg of muscle tissue was used for each specimen. The mitochondrial cytochrome c oxidase subunit I (COX1) gene was amplified using the universal primers FishF1 and FishR1 (Table 2). Each PCR reaction was performed in a total volume of 25 µl containing 14.3 µl sterile distilled water, 5 µl Taq buffer 5×, 2.0 µl of 25 mM MgCl, 0.5 µl of 10 mM dNTP, 0.5 µl of 10 µM of each primer, 0.2 µl of 5 µ µ-1 of Taq DNA polymerase

Table 2
Primer used in this study and the sequences

Primer	Sequences
FishF1	5'-TCAACCAACCACAAAGACATTGGCAC-3'
FishR1	5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'

*Source: Ward et al. (2005)

and 2 µl of DNA template. Amplification was carried out using a Mastercycler Gradient PCR system (Eppendorf, Hamburg, Germany) under the following cycling conditions: initial denaturation at 94 °C for 3 minutes, followed by 32 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 1 minute, and extension at 72 °C for 1 minute 30 seconds, with a final extension at 72 °C for 2 minutes. The amplified PCR products were separated on a 1% agarose gel stained with ethidium bromide and visualised under UV illumination using an AlphaImager HP system (BioTechne, USA). A BenchTop 1kb DNA Ladder (Promega Corp, Madison, USA) was used as the molecular size marker. PCR products that yielded clear bands of the expected size were purified prior to sequencing and subsequently submitted to First BASE Laboratories Sdn. Bhd. for forward-direction sequencing using an ABI 377 automated sequencer (Applied Biosystems).

Data Analyses

The phylogenetic analysis was constructed with the inclusion of two sequences from GenBank (MF622020 and MW381290). The Kimura 2-parameter evolution model was used to calculate sequence divergence, sequences were grouped by using the Neighbour-Joining method (Figure 1) and bootstrapped with 1000 replications using MEGA version 7 (Kumar et al., 2016). Genetic structure of the GFP was obtained from the summary statistics of Tajima's D and Fu's Fs using ARLEQUIN version 3.0 (Excoffier et al., 2005). The mismatch distribution analysis was performed to detect recent population expansion by comparing the distribution of the number of pairwise differences between population and their theoretical distribution expected under a model of sudden (stepwise) demographic expansion. In the mismatch distribution analysis, SSD (Sum of Squared Deviations) and r statistics are used to assess the goodness-of-fit of the observed mismatch distribution to the expected distribution under specific demographic models. The SSD is a measure that quantifies the difference between the observed mismatch distribution and the expected distribution under a particular demographic model. The r statistic, also known as Harpending's raggedness index, is another measure used to assess the smoothness of the observed mismatch distribution.

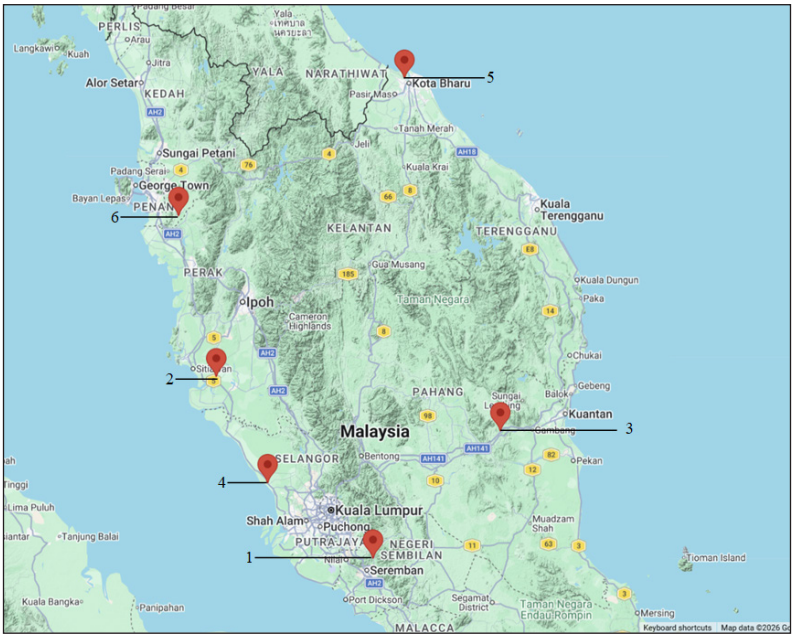

Figure 1. Sampling sites in Peninsular Malaysia; 1. Sungai Linggi, Negeri Sembilan; 2. Sungai Perak, Perak; 3. Sungai Pahang, Pahang; 4. Kuala Selangor, Selangor; 5. Sungai Selehong, Kelantan; 6. Sungai Kerian, Pulau Pinang

RESULTS

Sequence Characteristics and Haplotype Composition

A total of 572 base pairs (bp) of the COX1 gene were successfully retrieved after sequence alignment. 24 sites (4.19%) were identified as variable, including 15 parsimoniously informative sites, while the remaining 548 sites (95.8%) were conserved. The detected haplotypes contained eight substitutions, consisting of seven transitions and one transversion. The mean nucleotide composition was as follows: A = 27.3%, T = 27.6%, C = 26.9% and G = 18.2%. From the 149 GFP samples analysed, 12 distinct haplotypes were identified. All identified haplotypes were deposited in GenBank under accession numbers (PP337789- PP337800).

Genetic Diversity and Population Variation

Genetic diversity analysis revealed that GFP from Sungai Selehong (Kelantan) exhibited the highest genetic richness, with a haplotype diversity (h) of 0.5885 along with a relatively low nucleotide diversity (π = 0.0024). In contrast, the GFP from Sungai Perak (Perak) population displayed no genetic variation (h = 0.0000; π = 0.0000), with both haplotype and nucleotide diversity values equal to zero (Table 3), indicating a state of genetic homogeneity in that river system.

Table 3
Distribution of 12 observed haplotypes, nucleotide diversity, number of haplotypes, haplotype diversity and number of polymorphic sites among populations of GFP

Haplotypes	GenBank Accession Numbers	Populations					
		N. Sembilan (20)	Perak (29)	Pahang (23)	Selangor (24)	Kelantan (30)	Penang (23)
Hap 1	PP337789	0.45	-	-	-	-	-
Hap 2	PP337790	0.50	1.00	-	0.83	0.63	-
Hap 3	PP337791	0.05	-	0.04	0.13	0.10	-
Hap 4	PP337792	-	-	0.83	-	-	-
Hap 5	PP337793	-	-	0.04	-	-	-
Hap 6	PP337794	-	-	0.04	-	-	-
Hap 7	PP337795	-	-	0.04	-	-	-
Hap 8	PP337796	-	-	-	-	0.10	-
Hap 9	PP337797	-	-	-	-	0.07	-
Hap 10	PP337798	-	-	-	-	0.07	-
Hap 11	PP337799	-	-	-	-	0.03	-
Hap 12	PP337800	-	-	-	-	-	0.04
Nucleotide Diversity (PiJC)		0.0011	0	0.002	0.0004	0.0024	0.0007
Number of Haplotypes		3	1	5	2	6	3
Haplotype Diversity (Hd)		0.57368	0	0.3241	0.22826	0.58851	0.4229
Number of Polymorphic Sites		2	0	13	1	13	2

Phylogenetic Relationships among Populations

Phylogenetic analysis based on the Neighbour-Joining (NJ) tree revealed two distinct clades with high bootstrap values (Figure 2). The first clade (L1) contains 11 haplotypes, representing 148 samples, along with GFP sequences from Thailand available in GenBank. The second clade (L2) consisted of a single haplotype, Haplotype 11, which was exclusive to a single sample from Kelantan. Among the 12 identified haplotypes, five were unique to specific populations: Hap 5, Hap 6 and Hap 7 from Pahang, Hap 11 from Kelantan and Hap 12 from Pulau Pinang. The greatest genetic distance (2.9%) was observed between Hap 11 (Kelantan) and Hap 5/Hap 7 (Pahang), while the smallest distance was recorded between Hap 6 and Hap 4 (Table 4).

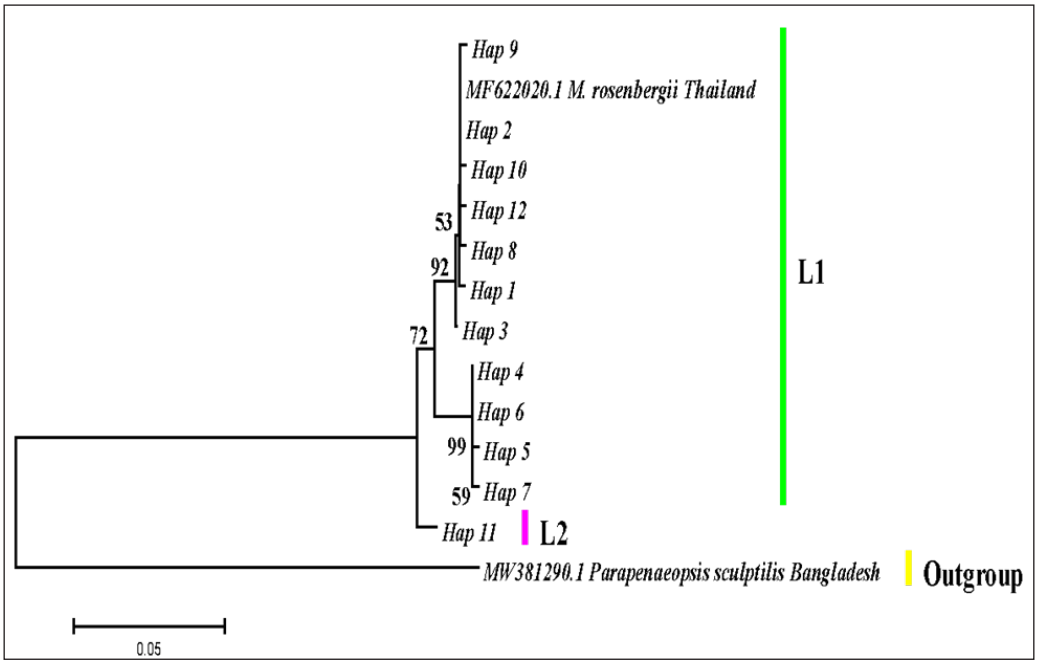

Figure 2. Neighbour-joining (NJ) tree showing relationships among the seabasses. The number at each node represents the bootstrap value (%) based on the 1000 pseudoreplications of the dataset

Genetic Differentiation Among Populations

Pairwise F_{st} values and results of the Chi-square tests for genetic differentiation among the GFP populations were presented in Table 5. However, there were no significant genetic differences among Peninsular Malaysia populations except samples from Sungai Linggi (Negeri Sembilan) and Sungai Pahang (Pahang) with the other populations in Peninsular Malaysia. AMOVA results (Table 6) revealed that the majority of the variance was from inter-population (83.34%), and only 16.66% of the total resulted from intra-population differentiation. The neutrality test of Tajima's D and Fu's Fs analysis was represented in Table 7. Negative Tajima's D was observed in all populations except Sungai Linggi (Negeri Sembilan) and Sungai Perak (Perak). However, only Sungai Pahang (Pahang), Sungai Selehong (Kelantan) and Sungai Kerian (Pulau Pinang) depicted a negative value for Fu's Fs test. The mismatch distribution was generally displayed as a multimodal pattern for all populations (Figure 3).

Table 4
Pairwise Tamura-Nei genetic distance among 12 haplotypes of GFP, one haplotype of Thailand GFP and one haplotype of Parapenaeopsis sculptilis

Haplotypes	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Hap 1	-													
Hap 2	0.002	-												
Hap 3	0.004	0.002	-											
Hap 4	0.023	0.021	0.019	-										
Hap 5	0.025	0.023	0.021	0.002	-									
Hap 6	0.023	0.021	0.019	0.000	0.002	-								
Hap 7	0.025	0.023	0.021	0.002	0.004	0.002	-							
Hap 8	0.004	0.002	0.004	0.023	0.025	0.023	0.025	-						
Hap 9	0.004	0.002	0.004	0.023	0.025	0.023	0.025	0.004	-					
Hap 10	0.004	0.002	0.004	0.023	0.025	0.023	0.025	0.004	0.004	-				
Hap 11	0.019	0.021	0.019	0.027	0.029	0.027	0.029	0.023	0.023	0.021	-			
Hap 12	0.004	0.002	0.004	0.023	0.025	0.023	0.025	0.004	0.004	0.004	0.023	-		
Macrobrachium rosenbergii	0.002	0.000	0.002	0.021	0.023	0.021	0.023	0.002	0.002	0.002	0.021	0.002	-	
Parapenaeopsis sculptilis	0.308	0.308	0.308	0.308	0.311	0.308	0.312	0.306	0.311	0.311	0.301	0.308	0.308	-

Table 5
Population subdivision (F_{st}) values and Chi-square probability test for population differentiation based on 1000 permutations of the sequence dataset among six populations of GFP

	1. N. Sembilan	2. Perak	3. Pahang	4. Selangor	5. Kelantan	6. P. Pinang
1	-					
2	0.436*	-				
3	0.920*	0.953*	-			
4	0.318*	0.102ns	0.937*	-		
5	0.137*	0.038*	0.886*	0.000ns	-	
6	0.283*	0.176*	0.925*	-0.011ns	0.00ns	-

Note. *$p < 0.001$; ns, not significant

Table 6
Hierarchical analysis of molecular variance (AMOVA) among populations GFP

Source of Variation	Sum of Squares	Variance Components	Percentage of Variation
Among Populations	197.718	1.58601	83.34
Within Populations	45.329	0.31699	16.66

Table 7
Mismatch distribution parameters, Tajima's D and Fu's FS neutrality tests of GFP from six different populations

	Tajima's D Test		Fu's FS Test		Mismatch Distribution (95% Confidence Intervals)	
Populations	Tajima's D	Tajima's D p-value	Fu's FS	Fu's FS p-value	r	SSD
N. Sembilan	0.2384	0.6970	0.2037	0.4560	0.2416	0.0376
Perak	0.0000	1.0000	0.0000	N. A.	0.0000	0.0000
Pahang	-2.3796	0.0010*	-0.6216	0.3230	0.2585	0.0095
Selangor	-0.2484	0.3090	0.2301	0.3060	0.3475	0.3005
Kelantan	-1.9489	0.0080*	-0.7737	0.3190	0.1348	0.0148
P. Pinang	-0.4084	0.3690	-0.3448	0.2600	0.1776	0.0118
Mean	-0.7912	0.3973	-0.2177	N. A.	0.1934	0.0624
Standard Deviation	0.9992	0.3583	0.3903	N. A.	0.1091	0.1071

Note. *Indicates significant level $p < 0.05$. Tajima's D and Fu's Fs are both neutrality test values

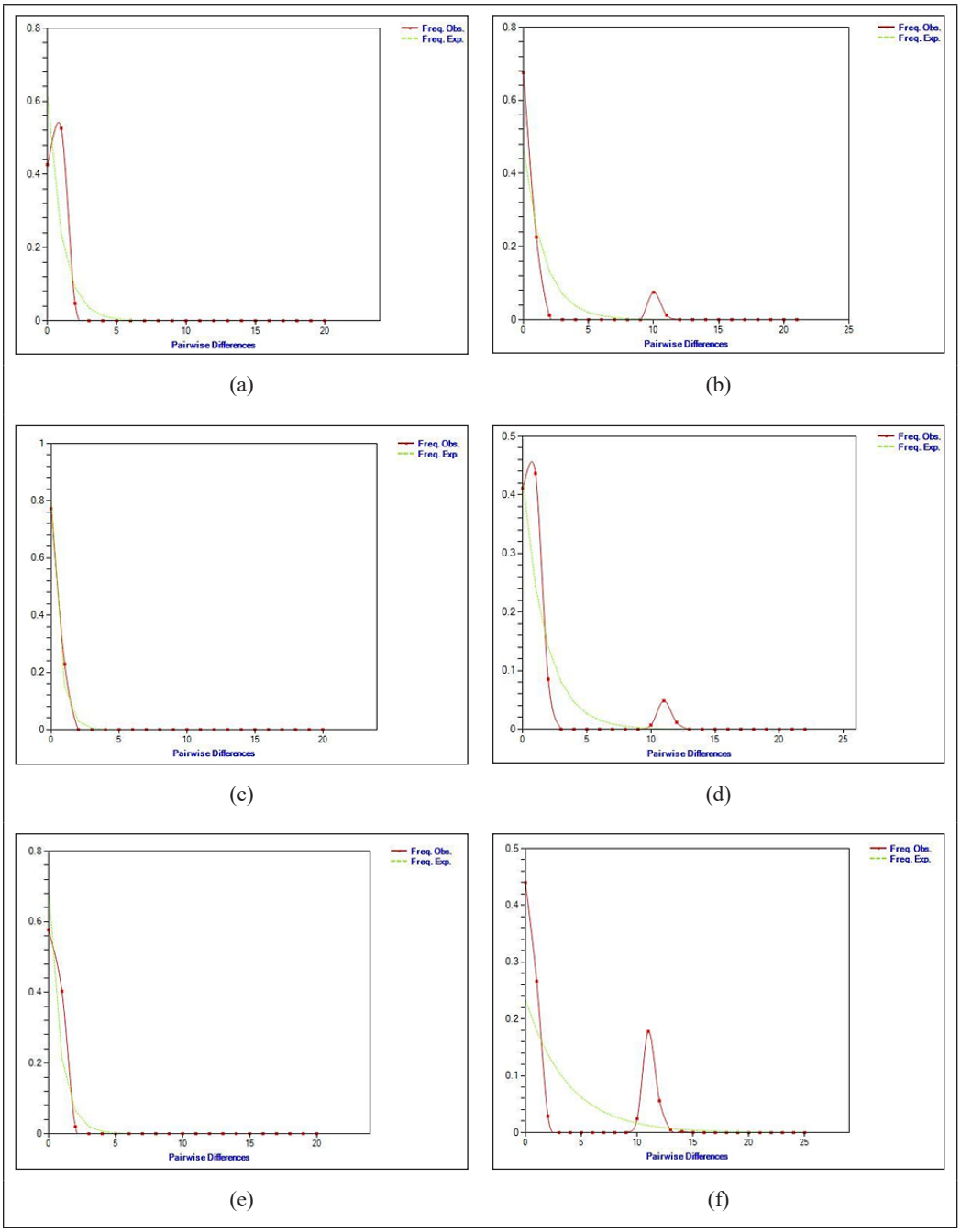

Figure 3. Mismatch distribution graphs of GFP for the: (a) N. Sembilan; (b) Pahang; (c) Selangor; (d) Kelantan; (e) P. Pinang; and (f) Total Populations

DISCUSSION

Phylogeography and Genetic Structure

The cytochrome c oxidase 1 (COX1) gene of the Giant Freshwater Prawn (*Macrobrachium rosenbergii*) was successfully sequenced from all samples collected from six distinct rivers across Malaysia. Among the populations examined, Sungai Selehong (Kelantan) exhibited the highest genetic variation. This variation was assessed through two primary genetic markers: nucleotide diversity (π) and haplotype diversity (h) (Senanan et al., 2015). Despite Sungai Selehong (Kelantan) population showing higher values of both π and h compared to the other populations, it was classified into the second category of population history, which is characterised by high haplotype diversity and low nucleotide diversity, suggesting a recent population expansion following a historical bottleneck (Grant & Bowen, 1998). Furthermore, the Java Island cluster, encompassing the Tabuk River and Peureulak populations, was assorted as a distinct group in genetics and became an isolated group, notably due to the significant genetic distance between populations. This fact is strengthened by the complete absence of shared haplotypes among the other four studied populations, which shows evidence of prolonged isolation and restricted gene flow. These are likely influenced by geographical separation and also the demographic history of the populations. These results emphasise the necessity for region-specific conservation and broodstock management to maintain the genetic integrity and adaptive potential of *M. rosenbergii* populations (Aliah et al., 2022).

A period of low effective population size, followed by a rapid expansion phase, is probably the cause of the separation. This result is consistent with the findings by Rogers and Herpending (1992), who found that accelerated population growth facilitated the retention of new mutations. This demographic signature generally features a dominant ancestral haplotype surrounded by peripheral haplotypes and often described as "tip" haplotypes in a star-like network. These peripheral haplotypes (Hap 11) represent recent mutational derivatives that differ by one or two nucleotide substitutions from the central, high-frequency haplotype (e.g. Hap 3). Given this, a phylogenetic analysis was constructed using a Neighbour-Joining (NJ) tree with high bootstrap support, and the results revealed that the new mutation, L2, was entirely composed of the unique haplotype Hap 11. This aligns with the previous assumption, thus within the L1 population, further examination of subclusters identified population-specific haplotypes, such as Hap 5, Hap 6 and Hap 7. Das et al. (2018) suggested that the emergence of these unique haplotypes is a result of the independent mutation events that occur within populations, increasing their genetic diversity.

Significant levels of both intra- and inter-population genetic variation were observed across the other five Malaysian river systems (Terengganu, Kelantan, Pahang, Selangor, and Negeri Sembilan), especially the Sungai Selehong (Kelantan) population, which

results demonstrate a high intra-population diversity and are also characterised by elevated nucleotide diversity (π) and haplotype diversity (h). This is in contrast to the genetic homogeneity observed in the Perak population, which shows otherwise. Moreover, this demographic signature aligns with a star-like phylogeny, where a high frequency of unique peripheral (tip) haplotypes (Hap 11) cluster around a central, ancestral haplotype (Hap 3). Thus, following a historical bottleneck, this pattern suggests a rapid population expansion, where accelerated growth facilitated the retention of new mutational derivatives. Contrary to previous findings, this study showed that the Perak population appears to be constrained by purifying selection and limited effective population size (Khan et al., 2021; Zhang et al., 2021), whereas the diversity in the other five rivers suggests a higher adaptive potential and a vigorous response to environmental changes. This result is consistent with that of a study by Whiteley et al. (2015), who reported that the gene flow through mechanisms such as genetic rescue initiatives and evolutionary rescue has been proposed as a strategy to maintain the viability of small, isolated populations. Despite this limitation, genetic rescue can help mitigate the effects of deleterious alleles that contribute to the genetic load in the gene pool, thereby promoting population growth, while evolutionary rescue enhances genetic variation, supporting long-term viability of *M. rosenbergii* in rivers across Peninsular Malaysia (Hoffmann et al., 2021).

In addition, genetic differences between haplotypes were found to be minimal, ranging from 0% to 0.2%, which suggests this may arise from mutations among closely related species. While the observed trend showed minimal genetic divergence between haplotypes, it was suggested that shallow evolutionary history, indicating that these lineages could possibly have only recently diverged from a common ancestor. Consequently, *M. rosenbergii* likely has a high degree of sequence similarity across different geographical locations in Peninsular Malaysia, and this reflects recent common ancestry or ongoing gene flow that has prevented significant genetic drift. Although significant genetic differentiation was observed between populations ($p<0.05$), the consistent variations in haplotypes, coupled with elevated Fst values, suggest minimal or no gene flow between geographically distant populations or those separated by extensive river systems. The lack of migration between these populations is further evidenced by the substantial population subdivision, which is consistent with the idea that migration plays a critical role in shaping genetic structure (Natasha et al., 2022).

Neutrality and Mismatch Distribution Tests

Neutrality tests and mismatch distribution are two effective tools used to study the demographic events in populations (Mousset et al., 2004). A stable population is typically indicated when the value of the neutrality test approaches zero. In addition, populations with negative and statistically significant neutrality test values are often considered to

have undergone historical expansion (Guo et al., 2023). Tajima's D test emphasises old mutations, while Fu's Fs test is more sensitive to recent mutation (Gunyakti Kilinc et al., 2020). The negative Tajima's D values observed in Pahang, Selangor, Kelantan, and Pulau Pinang suggest a history of population expansion, with an excess of rare nucleotide variants. The negative value observed in Fu's Fs test further supports this claim, except for the Selangor population, which displayed a positive value. These genetic patterns may be attributed to the founder effect (Dutta et al., 2020).

Significant negative Tajima's D values for Kelantan and Pahang populations were supported by the stable multimodal mismatch distribution graphs as shown in Figure 3, which suggest strong genetic subdivision and a stable population size, indicating demographic equilibrium (Gupta et al., 2018). While genetic hitchhiking could contribute to these patterns, the negative values in the neutrality tests seem indicative of purifying selection counterbalancing the genetic variation (Hills et al., 2008; Ruiz-Pesini et al., 2004). In contrast, unimodal mismatch distributions observed in the Selangor and Pulau Pinang populations rejected the neutrality test results. Therefore, this deviation may be due to recent population expansion events that may occur in these regions (Yan et al., 2023).

In Peninsular Malaysia, the characterisation of the phylogeography and genetic structure of *M. rosenbergii* offers a critical framework for transitioning from the traditional backyard aquaculture to a knowledgeable, genetically informed aquaculture. Therefore, by identifying the two distinct genetic clusters (L1 and L2) in this study, the results offer a baseline biological roadmap for the development of superior domestic broodstock, which farmers and industries could use as a guide. Consequently, breeders can leverage this spatial genetic information to implement strategic hybridisation of the species for production and potentially utilise heterosis to enhance commercially desirable traits such as growth rate, feed conversion efficiency, and disease resistance. Furthermore, the identification of unique haplotypes provides a molecular identification that allows for traceability and standardisation of specific lineages within closed-cycle hatchery systems, in turn reducing the industry's chronic reliance on unverified wild-caught postlarvae. This approach will assist in maintaining the genetic variations in the gene pool.

However, the findings still serve as an essential diagnostic tool beyond productivity for industry sustainability and risk mitigation. The observation from the Perak population highlights a significant vulnerability to inbreeding depression, possibly due to notably low genetic diversity in specific populations, which typically manifests as increased larval mortality, stunted and deformed growth and reduced environmental tolerance. In a world where climate change occurs annually, aquaculture management must prioritise the maintenance of genetic variance by avoiding the over-utilisation of broodstocks from bottlenecked populations and instead incorporating diverse genetic material from identified high-diversity population clusters to increase the heterozygosity and ensure long-term

viability. Future research should focus more on the development of climate-resilient strains that are capable of withstanding fluctuating water temperatures and emerging pathogens. This essential approach called genetic "insurance" is vital to realise the effort from shifting traditional aquaculture to develop a national breeding programme, which will harmonise conservation efforts by integrating phylogeographic insights with commercial objectives. This will ensure that the GFP will remain a robust and reliable food source despite the ever-changing ecological landscape.

CONCLUSION

The present study underscores the critical importance of understanding the genetic diversity and population structure of *Macrobrachium rosenbergii* populations for effective breeding programmes, aquaculture management and conservation. Generally, the findings revealed a significant genetic differentiation between two clusters (L1 and L2), likely driven by historical events. Notably, a major concern arising from this study is the low genetic diversity in populations such as Perak, which could pose a significant threat to their long-term viability. The identification of unique haplotypes and the observation of mutation accumulation provide valuable insights into the species' evolutionary processes. Concurrently, it should be noted that future research needs to prioritise phylogeographical studies and conservation strategies supporting the long-term resilience of *M. rosenbergii* populations to ensure the sustainability of this important species and enhance aquaculture breeding programmes.

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Application of Multi-Level Factorial Design in Optimisation of Ultrasound-assisted Extraction (UAE) Condition from *Erythrina variegata* L. Leaves on Antioxidant Activities

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ABSTRACT

Erythrina variegata L. (dedap), from the Fabaceae family, has been widely used as an anti-inflammatory, antimicrobial, antioxidant and wound healing agent. There is a lack of literature data regarding the optimisation of extraction parameters from this plant on antioxidant activities, particularly involving Ultrasound-assisted Extraction (UAE). Therefore, this study aims to determine the optimum extraction parameter for the UAE technique from *E. variegata* leaves extracts on in-vitro antioxidant activities by applying a multi-level factorial design. The solvent (methanol) concentration (X_1) and extraction time (X_2) were the independent variables, while the dependent variables are antioxidant assays: DPPH radical scavenging activity (Y_1), ferric reducing antioxidant power (FRAP) (Y_2) and total phenolic content (TPC) (Y_3). The results showed that the optimised extraction condition was at 100% solvent concentration and 60 minutes of extraction time,

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which demonstrated 0.301 ± 0.013 mg/mL, 54.624 ± 0.143 mg $FeSO_4$/g and 29.433 ± 0.062 mg GAE/g of DPPH activity, FRAP assay and TPC values, respectively. However, the highest extraction yield of 18.56% was obtained when extracted at only 25% solvent concentration and 60 minutes. This research has provided basic scientific evidence that optimum UAE conditions for *E. variegata* are crucial to maximising antioxidant activity.

Keywords: Antioxidant, *Erythrina variegata* L., extraction parameters, multi-level factorial design, ultrasound-assisted extraction

INTRODUCTION

Medicinal plants have been acknowledged as an alternative treatment for many ailments, including those in the genus *Erythrina*. This genus comprises approximately 110 species and belongs to the family of Fabaceae (Amir et al., 2011). One of the genera *Erythrina* cultivated in Malaysia is *Erythrina variegata* L. (synonym *Erythrina indica* L.), which is also recognised as the "Indian coral tree" or "Tiger's claw" and is locally referred to as "dedap" in Malaysia. It is a fast-growing tree that originates mainly in tropical and subtropical regions of the Asia-Pacific (Keerthana et al., 2024). This species is characterised by its striking flowers, trifoliate leaves, and rapid growth, reaching heights of up to 60 feet. Different parts of the plant, such as leaves, barks and flowers, have been employed by traditional practitioners in folk medicine as a nervine sedative, antiasthmatic, antiepileptic, antiseptic, and astringent (Kavitha et al., 2023; Kumar et al., 2010). Due to its diverse pharmacological potential, *E. variegata* has become a subject of increasing interest for phytochemical and pharmacological research.

Species from the *Erythrina* genus have enormous sources of secondary metabolites, particularly flavonoids, isoflavones, and pterocarpans, which possess strong antioxidant properties (Jiménez-Cabrera et al., 2020). These compounds are effective in neutralising reactive oxygen species (ROS), scavenging free radicals, and inhibiting lipid peroxidation (Kavitha et al., 2023; Sakat & Juvekar, 2010). Antioxidants derived from *E. variegata* have shown promising *in-vitro* activity, including high DPPH radical scavenging capacity, indicating their potential role in preventing oxidative stress-related ailments such as cardiovascular disorders, inflammation, and cancer (Kavitha et al., 2023; Laveena & Chandra, 2018). Plant-based compounds have drawn researchers' interest nowadays as potential alternatives for synthetic antioxidants due to their safety and low toxicity to vertebrates (Baranitharan et al., 2019). Consequently, further exploration of *E. variegata* as a natural antioxidant source may contribute to the development of safe, plant-based therapeutic agents.

The extraction process is a cornerstone in natural product research, which involves desired bioactive compounds that are separated from the raw material. Continuous initiatives are being made to obtain and discover better extraction techniques that are

more economical and efficient (Pandey et al., 2024). Extraction technique, solvent type, solvent concentration and also extraction time and extraction temperature will influence the extraction efficiency of bioactive compounds and antioxidant potential in the extract (Wong et al., 2014). In this study, ultrasound-assisted extraction is employed due to its proven efficiency in extracting phenolic and flavonoid compounds, which are key contributors to antioxidant activity (El Baakili et al., 2023). It is a modern extraction technique that applies high-frequency ultrasonic waves to create acoustic cavitation in the extraction medium, which can cause the disruption of plant cell walls and facilitate the drug surface area for solvent penetration (Demesa et al., 2024). Furthermore, the UAE method allows for fine-tuning of variables such as sonication time, amplitude, solvent concentration, and solid-to-liquid ratio. By optimising these parameters using statistical tools like response surface methodology (RSM), UAE can maximise extraction efficiency while minimising degradation of target compounds (Belwal et al., 2016; Damayanti et al., 2021). Despite the reported antioxidant potential of *E. variegata*, limited studies have focused on optimising extraction parameters using ultrasound-assisted extraction (UAE). In particular, there is a lack of systematic studies employing a multilevel factorial design to evaluate the interaction between extraction variables and their combined effect on antioxidant activity. Therefore, this study aims to address this gap by optimising UAE conditions (solvent concentration and extraction time) to maximise antioxidant recovery from *E. variegata* leaves by applying a multi-level factorial design.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents used were of analytical grade. Folin-Ciocalteu reagent, gallic acid monohydrate, 2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tri-2-pyridinyl-1,3,5-triazine (TPTZ), and hydrochloric acid were procured from Sigma Aldrich, Germany. Meanwhile, sodium carbonate anhydrous, sodium acetate trihydrate, acetic acid, iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), and iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$) were purchased from QRëC, Malaysia. Methanol and ascorbic acid were acquired from R&M, Malaysia, and Thermo Fisher, USA, respectively.

Sample Collection and Preparation

Fresh *Erythrina variegata* L. leaves were collected from a local cultivator at Jasin, Melaka, in December 2024. It is verified by Dr. Khairil Mahmud, a botanist from the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia (UPM), with the voucher specimen number (KM0116/24), and it was deposited at the Herbarium Unit, Universiti Putra Malaysia (UPM), for future reference. The leaves were cleaned, air-dried in the shade for around a week or until the moisture content reduced to about 10% (Rahim et al., 2022). It was then ground into powder and kept in sealed plastic bags.

Sample Extraction (Ultrasound-assisted Extraction (UAE))

The UAE procedure was conducted as reported by Zahari et al. (2025) with slight alterations. The powdered *E. variegata* leaves were mixed with methanol at various concentrations ranging from 0%, 25%, 50%, 75%, and 100% v/v, following a 1:10 ratio of solid to solvent. The mixture was then placed in an ultrasonic cleaner (Elma E-30-H, Germany) with a power of 280 W and frequency of 37 kHz setting for varying extraction times of 30, 45, and 60 minutes and at a fixed extraction temperature of 60 °C. The sample was then centrifuged for 15 minutes at 4200 rpm, followed by filtration using Whatman filter papers. The resulting filtrate was evaporated under reduced pressure at 60 °C with a rotary evaporator (Buchi Switzerland R-100, Switzerland) until a constant weight was obtained, and the extraction yield was calculated as in Equation 1. The crude extract was stored in a vial at -20 °C for further antioxidant testing.

$$\text{Extraction yield (\%)} = (W_{ext} / W_p) \times 100 \quad [1]$$

where W_p is the mass of the dry plant and W_{ext} is the mass of crude extract

The fixed conditions of ultrasonic power (280 W) and frequency (37 kHz) were selected based on the method by Zahari et al. (2025) of the ultrasonic bath, and previous studies also reported that effective cavitation within this range for phenolic extraction (Dzah et al., 2020; Gao et al., 2022). Meanwhile, the extraction temperature was fixed at 60 °C to enhance mass transfer while preventing thermal degradation of heat-sensitive bioactive compounds (Dzah et al., 2020). These parameters were kept constant to allow focused optimisation of solvent concentration and extraction time, which are known to significantly influence extraction efficiency. The solvent concentration range (0-100% methanol) was selected to represent a wide polarity spectrum, as solvent polarity significantly influences the extraction of phenolic compounds. Similarly, extraction time (30-60 minutes) was chosen based on commonly reported UAE durations that balance extraction efficiency and compound stability (Dzah et al., 2020; Gao et al., 2022; Kaushik & Barmanray, 2025).

Experimental Design

A multi-level factorial design was applied to determine the optimum UAE condition of *E. variegata* leaves extract to achieve maximum antioxidant activities. The multi-level design is a unique type of full factorial design that permits a random number of levels for the studied factors in an experiment, which encompasses every possible combination of factor level (Elkanayati et al., 2024). In this study, the solvent concentration (X_1) and extraction time (X_2) were the independent variables to optimise the response variables,

which are antioxidant assays: DPPH radical scavenging activity (Y_1), ferric reducing antioxidant power (FRAP) (Y_2) and total phenolic content (TPC) (Y_3). The independent variables (factors) and their varying levels are shown in Table 1. The experimental runs were built using a statistical software package, Design Expert® (Version 13, State Ease, Inc., Minneapolis, USA) and generated a total of 15 runs.

Table 1
Levels of independent variables used in the multilevel factorial design

Factors	Unit	Levels	Values
Solvent Concentration (X_1)	% (v/v)	5	0, 25, 50, 75, 100
Extraction Time (X_2)	min	3	30, 45, 60

Antioxidant Activity - DPPH Radical Scavenging Activity

The antioxidant activity of *E. variegata* leaves extract was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Itam et al., 2021) with minor changes. In this method, DPPH methanolic solution (0.1 mM) was prepared freshly, and 2 mL of the DPPH solution was mixed with the sample extract (1 mL). The mixture was thoroughly vortexed for about 1 minute and then kept in the dark for 30 minutes. The measurement of the absorbance reading was taken at 517 nm using a UV-Vis spectrophotometer (T60, PG Instruments, UK). The percentage of DPPH radical scavenging activity was calculated using the following Equation 2:

$$\text{DPPH radical scavenging activity (\%)} = [(A_0 - A_1) / A_0] \times 100 \quad [2]$$

where A_0 represents the absorbance of the control, and A_1 represents the absorbance of the extract or standard

Additionally, the DPPH radical scavenging activity of the positive control (ascorbic acid) and all *E. variegata* leaves extracts was evaluated and expressed as an IC_{50} value, which indicates the concentration of the extracts required to scavenge 50% of the radicals. Five different concentrations varying from 0.1, 0.25, 0.5, 1 and 2 mg/mL of each sample were prepared, and the IC_{50} value was determined through linear regression between inhibition percentage and sample concentration. The IC_{50} value classified antioxidant activity as follows: IC_{50} < 50 µg/mL (very strong), IC_{50} = 50-100 µg/mL (strong), IC_{50} = 101-250 µg/mL (moderate), IC_{50} = 250-500 µg/mL (weak), and IC_{50} > 500 µg/mL (inactive) (Itam et al., 2021).

Antioxidant Activity - Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was performed according to the procedure described by Chaves et al. (2020) with minor changes. *E. variegata* leaves extract (0.2 mL) was mixed with FRAP reagent (3.8 mL). The FRAP reagent was freshly prepared by combining 1 part of 10 mM TPTZ, 1 part of 20 mM $FeCl_3 \cdot 6H_2O$ and 10 parts of 300 mM sodium acetate buffer (pH 3.6). The mixture was then incubated in the dark at 37°C for 10 minutes. After incubation, the absorbance reading was assessed at 593 nm using a UV-Vis spectrophotometer. A blank sample was prepared by replacing the extract with an equal volume of methanol. The FRAP results were expressed as milligram equivalents of $FeSO_4$ per milligram of dry weight. A standard calibration curve was plotted using $FeSO_4$ solutions at different concentrations (20, 40, 60, 80, and 100 mg/mL).

Total Phenolic Content (TPC)

The total phenolic content in the extracts was analysed using the Folin-Ciocalteu colourimetric method with minor modifications (Proestos et al., 2013). Briefly, *E. variegata* leaves extract (500 µL) was added to a volumetric flask sized 10 mL that contained 2.5 mL of Folin-Ciocalteu reagent (diluted 10 times and freshly prepared) and left in the dark for 5 minutes at ambient temperature. Then, 2 mL of sodium carbonate (7.5% w/v) was added, and the flask was filled to volume with distilled water. The mixture was shaken vigorously and allowed to stand in the dark for 120 minutes. The absorbance was then measured at 760 nm using a UV-Vis spectrophotometer. Calibration was performed using a standard curve prepared with various concentrations (0.02, 0.04, 0.06, 0.08, and 0.10 mg/mL) of gallic acid, and the results were expressed as milligrams of gallic acid equivalent (mg GAE) per gram of extract. All measurements were conducted in triplicate for each extract.

Statistical Analysis

All analyses were carried out in triplicate, and the findings were conveyed as means ± standard deviation (SD). Differences between the variables were evaluated for significance using the one-way ANOVA with Tukey-LSD analysis procedure when $p<0.05$. All analyses were performed using OriginPro 2018 software (Aranda-Ledesma et al., 2024).

RESULTS AND DISCUSSION

Extraction Yield

Table 2 shows the results of the extraction yield obtained for each trial run generated on the ultrasound-assisted extraction condition. Extraction yield was measured by dividing the mass of extract by the mass of dry matter to determine the effect of extraction efficiency on *E. variegata* leaves extract.

Table 2
Extraction yield obtained from E. variegata *leaves extract*

Run No.	Extraction Parameters		Extraction Yield (%)
	Solvent (Methanol) Concentration (%)	Extraction Time (min)	
1	0	30	13.00
2	25	30	16.00
3	50	30	17.44
4	75	30	15.92
5	100	30	5.56
6	0	45	5.20
7	25	45	15.00
8	50	45	17.48
9	75	45	10.52
10	100	45	7.08
11	0	60	12.08
12	25	60	18.56
13	50	60	18.24
14	75	60	9.40
15	100	60	8.52

The highest yield was obtained when extracted at 25% solvent concentration and 60 minutes (Run 12) with 18.56%. In this study, the solvent used was methanol, with varying from 0 to 100% concentration. The solvent concentration of 25% had the highest yield, which may be due to the solvent polarity increasing and improving solvent penetration to the plant matrix, allowing a wider range of compounds to be extracted (Gil-Martín et al., 2022). However, the extraction process may be disrupted if the solvent is too concentrated (Zhang et al., 2018). This is agreed in a previous study that stated the extraction yield for water-methanol is higher than that of organic solvents alone (Dhanani et al., 2017). In addition, prolonged extraction time at higher temperatures can often lead to greater extraction yields, but it can also lead to degradation of the extracted compounds. For this study, the extraction yield was higher when extracted for a longer time (60 minutes), but the temperature used was fixed at 60 °C. This condition maintains the composition of bioactive compounds, particularly polyphenols, which may be damaged at higher temperatures exceeding 60 °C as established in prior studies (Dzah et al., 2020; Hlatshwayo et al., 2025).

As mentioned previously, the higher extraction yield observed at lower solvent concentrations (25%) may be attributed to the extraction of highly polar compounds such as sugars, proteins, and other non-phenolic constituents. In contrast, higher solvent concentrations (100%) assist the extraction of less polar phenolic and flavonoid compounds, which are predominantly responsible for antioxidant activity.

This explains the observed discrepancy between extraction yield and antioxidant activity. Solvent polarity plays a critical role in determining the selectivity and efficiency of compound extraction, influencing the solubility of different phytochemicals (Lučić & Onjia, 2025). Furthermore, ultrasound-assisted extraction enhances the release of intracellular compounds through cavitation and cell wall disruption, improving the recovery of bioactive compounds (Shen et al., 2023). Similar findings have been reported where increased extraction yield does not necessarily correspond to higher bioactivity, particularly antioxidant activity, due to differences in extract composition and compound selectivity (Demesa et al., 2024).

Optimisation of Extraction Parameters - Model Fitting

A multi-level factorial design was employed to optimise the ultrasound-assisted extraction conditions of *E. variegata* leaves extracts for maximising the antioxidant activities. Table 3 showed the outcomes of the independent variables (solvent concentration, X_1; extraction time, X_2) and their influence on the response variables (antioxidant activities - DPPH, Y_1; FRAP, Y_2; TPC, Y_3) across 15 experimental runs on UAE. These three assays were chosen to comprehensively assess antioxidant potential, as it is suggested to perform at least two or more complementary assays due to the complexity of antioxidant mechanisms. The DPPH assay is a widely used method that evaluates the ability of antioxidants to neutralise free radicals by donating hydrogen atoms or electrons, thus reflecting free radical scavenging capacity (Gulcin & Alwasel, 2023). Meanwhile, FRAP measures the ability of an antioxidant to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions, indicating the reducing power and potential metal-chelating activity of the extract. The TPC assay basically quantifies the phenolic compounds that strongly correlated with antioxidant activity, as phenolics contribute significantly to the scavenging and reducing capacity of plant extracts (Chaves et al., 2020). The primary aim of this study was to optimise extraction parameters to maximise antioxidant activity and phenolic recovery, which is a common approach in UAE studies before detailed compound identification.

The table shows that the extraction parameters had a significant effect on antioxidant activity. Run 15 (solvent concentration of 100% and extraction 60 minutes) had significantly higher antioxidant activities (IC_{50}, 0.272 ± 0.003 mg/mL for DPPH; 54.624 ± 1.983 mg $FeSO_4$/g for FRAP) and TPC values (29.541 ± 0.306 mg GAE/g) as compared to other experimental runs. The enhanced antioxidant activity observed at higher methanol concentrations can be attributed to the selective extraction of phenolic compounds such as flavonoids, isoflavones, and phenolic acids, which are known to be soluble in organic solvents. Methanol has been widely reported as an effective solvent for extracting these compounds due to its polarity, which is able to penetrate plant matrices of *E. variegata* leaves extract better and leads to an enhancement of extraction efficiency

Table 3
Experimental design with the response variable of antioxidant activities

Run No.	Extraction Parameters		Antioxidant Activities		
	Solvent (Methanol) Concentration (%)	Extraction Time (min)	DPPH, IC_{50} (mg/mL)	FRAP (mg $FeSO_4$/g)	TPC (mg GAE/g extract)
Ascorbic Acid	-	-	0.189 ± 0.071^{a}	-	-
1	0	30	0.321 ± 0.002^{c}	29.249 ± 0.488^{d}	26.689 ± 0.501^{b}
2	25	30	0.490 ± 0.001^{d}	30.344 ± 2.100^{d}	28.276 ± 1.361^{a}
3	50	30	0.442 ± 0.004^{d}	32.628 ± 1.176^{d}	24.578 ± 1.391^{c}
4	75	30	0.421 ± 0.003^{d}	24.399 ± 0.236^{e}	27.711 ± 1.212^{b}
5	100	30	0.288 ± 0.003^{b}	35.225 ± 1.239^{c}	28.828 ± 1.142^{a}
6	0	45	0.357 ± 0.004^{c}	40.544 ± 0.482^{c}	14.544 ± 0.199^{e}
7	25	45	0.485 ± 0.002^{d}	32.253 ± 0.895^{d}	23.542 ± 0.511^{d}
8	50	45	0.461 ± 0.003^{d}	31.783 ± 2.397^{d}	24.161 ± 1.952^{c}
9	75	45	1.158 ± 0.004^{f}	33.911 ± 3.802^{d}	24.403 ± 0.458^{c}
10	100	45	1.755 ± 0.008^{f}	45.112 ± 5.541^{b}	25.734 ± 0.874^{c}
11	0	60	1.064 ± 0.001^{f}	37.447 ± 1.034^{d}	24.241 ± 0.176^{c}
12	25	60	0.377 ± 0.001^{c}	32.534 ± 5.216^{d}	28.451 ± 0.537^{a}
13	50	60	0.490 ± 0.001^{d}	44.799 ± 2.401^{b}	27.335 ± 0.269^{b}
14	75	60	0.516 ± 0.002^{e}	37.071 ± 1.067^{d}	26.366 ± 0.292^{b}
15	100	60	0.272 ± 0.003^{b}	54.624 ± 1.983^{a}	29.541 ± 0.306^{a}

Note. Different letters in each column indicate significant differences ($p<0.05$); DPPH = 2,2-diphenyl-1-picrylhydrazyl; FRAP = Ferric reducing antioxidant power; TPC = Total phenolic content; Ascorbic acid was used as a positive control for DPPH assay

for phenolics and antioxidants (Ahmed et al., 2024; Jiménez-Cabrera et al., 2020). The antioxidant activity of the extract was also compared with the positive control (ascorbic acid), which exhibited a moderate radical scavenging activity (IC_{50}, 0.189 ± 0.071 mg/mL), and it is well-known to act as a reducing agent that effectively reduces the DPPH radical (Jiménez-Cabrera et al., 2020).

In addition, a longer extraction time gives sufficient time for the penetration of phenolic compounds into the solvent and improves the antioxidant activities (Uma et al., 2010). However, a contradictory finding was observed for the lowest value of DPPH (run 10; solvent concentration 100% and 45 minutes), FRAP (run 4; concentration 75% and 30 minutes) and TPC (run 6; concentration 0% and 45 minutes). This may be due to the fact that the extraction time was inadequate to extract more antioxidant compounds (Mungwari et al., 2024). The UAE method generally involves the use of ultrasound that is significantly able to preserve the chemical integrity of bioactive compounds under standard conditions, which is important in maintaining the antioxidant properties of the extract (Demesa et al., 2024).

Table 4 depicts the analysis of variance (ANOVA) findings for the UAE condition on antioxidant activities. ANOVA was performed to reveal the significance of each independent variable (extraction parameters) and their interaction on the measured antioxidant responses. The reliability and adequacy of the model were evaluated using *p*-values and F-values, where low *p*-values ($p<0.05$) confirm the statistical relevance and high F-values indicate strong model significance (Pimsamarn et al., 2024; Prakoso et al., 2022). Based on the table, factors X_1 (solvent concentration), X_2 (extraction time) and X_1*X_2 interaction (extraction parameters condition) have a *p*-value of less than 0.05, denoting that both factors combined play a crucial role in determining antioxidant response. Furthermore, the model F-value obtained was high, with 6208.75 (DPPH), 175018.67 (FRAP), and 151651.35 (TPC), suggesting that the regression models were highly significant and not influenced by random variation. It should be noted that the possibility of this high F-value being caused by noise is only a 0.01% (Bayat & Shokri, 2021; Madhu et al., 2018). This indicates that the model for all antioxidant activities has a significant impact on the changes in the extraction conditions.

The model summary statistics for ultrasound-assisted extraction (UAE), including the coefficient of determination (R^2), adjusted R^2, predicted R^2, standard deviation (SD), mean, coefficient of variation (CV), and adequate precision, are shown in Table 5. The R^2 value is a main indicator of how well the experimental data fit the regression model, with values approaching 1.0 reflecting excellent model performance (Aguilar-Ascon et al., 2020). In this study, the R^2 obtained for DPPH, FRAP and TPC was 0.9898, 0.9856 and 0.9927, respectively, which reveals that up to 98% of the data fit the regression model for all assays studied.

Table 4
ANOVA analysis of extraction parameters on antioxidant activities

Source	DPPH					FRAP					TPC				
	SS	**DF**	**MS**	**F-value**	***p*-value**	**SS**	**DF**	**MS**	**F-value**	***p*-value**	**SS**	**DF**	**MS**	**F-value**	***p*-value**
Model	7.12	14	0.5085	6208.75	< 0.0001	2319.69	14	165.69	175018.67	< 0.0001	550.90	14	39.35	151651.35	< 0.0001
X_1	0.72	4	0.1797	21925.40	< 0.0001	912.20	4	228.05	$2.409e^{+05}$	< 0.0001	197.32	4	49.33	$1.901e^{+05}$	< 0.0001
X_2	1.58	2	0.7900	96357.15	< 0.0001	1002.54	2	501.27	$5.295e^{+05}$	< 0.0001	223.52	2	111.76	$4.307e^{+05}$	< 0.0001
X_1X_2	4.82	8	0.6026	73498.32	< 0.0001	404.95	8	50.62	53467.99	< 0.0001	130.06	8	16.26	62653.64	< 0.0001
Residual	0.0002	28	$8.198e^{-06}$			0.0265	28	0.0009			0.0073	28	0.0003		
Corrected Total	7.12	44				2319.73	44				550.91	44			

Note. DPPH = 2,2-diphenyl-1-picrylhydrazyl; FRAP = Ferric reducing antioxidant power; TPC = Total phenolic content; DF = degree of freedom; SS = Sum of squares; MS = Mean square; X_1 = solvent concentration (%), X_2 = extraction time (min)

Table 5
Model summary statistics of extraction parameters on antioxidant activities

Terms	DPPH	FRAP	TPC
R^2	0.9898	0.9856	0.9927
Adjusted R^2	0.9753	0.9714	0.9837
Predicted R^2	0.9666	0.9653	0.9638
Adequate Precision	845.28	1598.33	1517.44
Standard Deviation	0.00	0.03	0.02
Mean	0.59	36.40	25.63
CV (%)	0.48	0.09	0.06

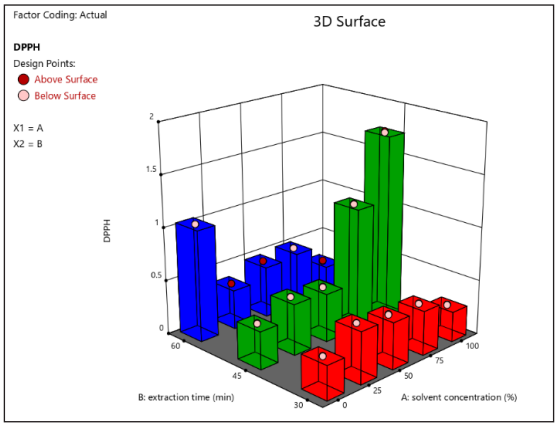
Note. DPPH = 2,2-diphenyl-1-picrylhydrazyl; FRAP = Ferric reducing antioxidant power; TPC = Total phenolic content; CV = Coefficient of variance

Moreover, the difference between predicted R^2 and adjusted R^2 was less than 0.2, indicating strong agreement between them. Meanwhile, adequate precision, which measures the signal-to-noise ratio of the model, further confirmed the reliability of the results. The adequate precision values obtained were 845.28 (DPPH), 1598.33 (FRAP), and 1517.44 (TPC), which are above the minimum acceptable threshold of 4.0 (Bayat & Shokri, 2021). Besides that, the obtained CVs were 0.48% (DPPH), 0.09% (FRAP), 0.06% (TPC) and these values were below 10% that implies good precision and reliability of the experiments (Rahim et al., 2022).

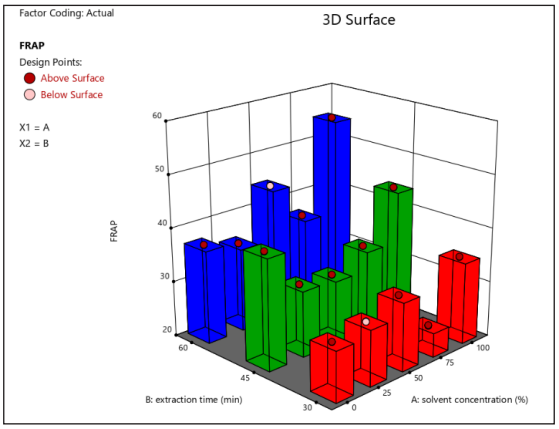
Effect of Extraction Parameters on Antioxidant Activities

Figure 1 illustrates the 3D surface plot of the independent variables (solvent concentration and extraction time) on the antioxidant activities, where Figure 1a - DPPH; Figure 1b - FRAP; Figure 1c - TPC for ultrasound-assisted extraction (UAE). For the DPPH activity, the results showed that when extracted at 100% of solvent concentration and 60 minutes of extraction time, it had a low IC_{50} value of 0.272 mg/mL, which indicates high antioxidant activity. Similar results were shown on FRAP and TPC, where, under the same conditions, they revealed a potential FRAP activity of more than 50 mg $FeSO_4$/g and almost 30 mg GAE/g of TPC values. This agrees with a previous study that stated an optimal condition for UAE is likely around higher solvent concentration (75% to 100%) and longer extraction times (Wang et al., 2013).

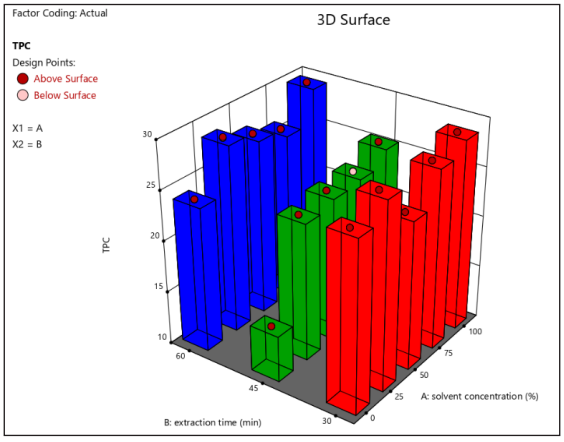
From Figure 1, the improved extraction efficiency observed in this study can be explained by the mechanism of UAE cavitation. During the UAE, ultrasonic waves generate microbubbles that undergo rapid growth and violent collapse, producing localised high pressure, temperature, and microjets. These effects disrupt plant cell walls, increase solvent penetration, and enhance the release of intracellular bioactive compounds (Demesa et al., 2024; Shen et al., 2023).



(a)



(b)



(c)

Figure 1. 3D response surface plots of *E. variegata* leaves extract showing the effect of extraction parameter conditions for ultrasound-assisted extraction (UAE) on (a) DPPH; (b) FRAP; and (c) TPC

Earlier research demonstrated that direct contact between sound waves in the UAE technique and the extraction medium allows better interaction and elevates the extraction of target compounds from cellular plant matrices (Dzah et al., 2020). This method has also demonstrated a significant influence on the bioactivity of phenolic compounds by considering the solvent used, temperature, and duration of extraction (Ramesh et al., 2024). Previous studies have demonstrated that UAE yields higher phenolic content and antioxidant activity when compared to conventional methods, such as maceration and Soxhlet extraction, primarily due to reduced thermal degradation and enhanced mass transfer (Ahmed et al., 2024; Ramesh et al., 2024).

Model Validation

The optimum UAE condition was verified by utilising the desirability of the responses using the statistical software. Table 6 displayed the summary of the validation process on the antioxidant activities of *E. variegata* leaves extract. The optimised UAE condition was at 100% solvent concentration (X_1) and 60 minutes of extraction time (X_2) and exhibited an IC_{50} value of 0.301±0.013 mg/mL for DPPH radical scavenging activity (Y_1), 54.624 ± 0.143 mg $FeSO_4$/g of FRAP values (Y_2) and 29.433 ± 0.062 mg GAE/g of TPC values (Y_3). This is similar to a previous study on *Mesembryanthemum edule* L. shoots that showed high phenolic and antioxidant properties when extracted only with 100% methanol (Dzah et al., 2020; Falleh et al., 2012). The experimental values should match the predicted values under this optimal condition, with the percentage of errors being less than 10% (Bohui et al., 2024). The results also presented that there is no significant difference (p>0.05) between the predicted and experimental findings of the antioxidant activities. This proves that the application of a multi-level factorial design, which involves a design with variable factor levels, is valid for predicting the antioxidant activities in the selected range of extraction conditions.

Table 6
Summary of the validation process on both extraction techniques on the antioxidant activities

Antioxidant Activities	Predicted Value	Experimental Value	Percentage of Error (%)
DPPH, IC_{50} (mg/mL)	0.272	0.301 ± 0.013[a]	9.74
FRAP (mg FeSO4/g)	54.666	54.624 ± 0.143[a]	0.08
TPC (mg GAE/g)	29.552	29.433 ± 0.062[a]	0.40

Note. DPPH = 2,2-diphenyl-1-picrylhydrazyl (DPPH); FRAP = Ferric reducing antioxidant power; TPC = Total phenolic content

CONCLUSION

In summary, optimisation of extraction conditions for *Erythrina variegata* leaves extracts was successfully carried out using a multi-level factorial design for ultrasound-assisted extraction (UAE). Design-Expert software enabled accurate prediction and validation of responses for all antioxidant activities (DPPH, FRAP and TPC). The optimised extraction condition was at 100% solvent concentration (X_1) and 60 minutes of extraction time (X_2), which exhibited an IC_{50} value of 0.301±0.013 mg/mL for DPPH radical scavenging activity (Y_1), 54.624 ± 0.143 mg $FeSO_4$/g of FRAP values (Y_2) and 29.433 ± 0.062 mg GAE/g of TPC values (Y_3). This indicated that the UAE method has high extraction efficiency in terms of extraction yields (18.56%) and across all measured antioxidant activities in *E. variegata* leaves extracts. This method is straightforward, less time-consuming, and relatively cheaper than other conventional extraction methods, such as maceration and Soxhlet extraction. Therefore, it can be summarised that the identified optimised UAE conditions could have potential for large-scale application in tropical agricultural processing, particularly in the development of products involving *E. variegata* leaves extracts in a food or nutraceutical industry, as this method is considered a scalable and efficient technique. Future work involving LC-MS/MS analysis is recommended to further elucidate the specific compounds responsible and establish a clearer relationship between extraction conditions and metabolite composition.

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Review Article

Life Cycle and Feeding Behaviour of Rice Stem Borer Species in Southeast Asian Regions' Paddy Fields

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ABSTRACT

Rice stem borers are among the most damaging pests in paddy fields and are a growing concern for farmers. This is concerning due to the decline in rice production they may cause, especially in areas with high infestation, where infestation coincides with critical stages of paddy growth. This review discusses existing knowledge on the life cycle, feeding behaviour, and oviposition patterns of six main recognised species in Southeast Asian regions, particularly *Scirpophaga incertulas*, *Scirpophaga innotata*, *Chilo suppressalis*, *Chilo polychrysus*, *Chilo auricilius*, and *Sesamia inferens*. Their distinct characteristics in egg-laying behaviour, feeding sites, and the infestation timing were then further

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discussed and correlated with rice phenology to observe the variation of damage expression and how it affects yield, ultimately. Though common symptoms such as deadheart and whitehead are expected after infestation, the occurrence and severity of damage, as well as the chances of plant recovery, may differ. The differences between the infestation events are crucial, especially when considering management controls in the field to produce better alignment with paddy growth stages, together with suitable ecological factors. However, there is still limited information on specific economic thresholds in many parts of Southeast Asia. This research gap provides the need for future research, as it may support more targeted IPM control strategies and reduce dependence of farmers on broad-spectrum insecticides.

Keywords: Feeding behaviour, life cycle, oviposition, phenology, rice stem borer, Southeast Asia

INTRODUCTION

These days, rice cultivation around the world faces many challenges, including harmful pests that lead to significant yield losses. 120-200 million tons of annual loss in rice production is attributed to insect pests, approximately 37% of the average global yield (Song et al., 2025). Among these factors, one of the most damaging insect pests in rice cultivation is the rice stem borer, which is considered one of the most economically important insect pests. This particular pest is a concerning issue due to its damaging feeding patterns, distribution, and continuous infestation across multiple growth stages, which makes it difficult to control among rice farmers.

The focus of this review is on existing stem borer species in the Southeast Asia region, where rice production is one of the backbones of agricultural economies, as well as serving as a staple food for the people. Most of them are rice-producing countries and consumers, making rice an important food source for their people. Any yield losses in this sector, particularly from stem borer, may lead to detrimental impacts on food availability and production stability, especially for those countries that rely on imports as their agricultural economies.

Rice stem borers around the world have been identified as approximately 70 species, including pyralid, noctuid, and diopsid taxa. This pest remains a persistent challenge in Southeast Asia, especially for the local or rural farmers. This is due to the damage done by the pest, which may lead to a reduction in tiller survival, development of paddy panicle, and eventually disruption in grain yield production. Over generations, they are renowned as a significant pest, yet most studies have examined individual species in isolation or have emphasised population density and yield loss relationships, often treating the pest as internal feeders that infest in the same way.

In this review, the emphasis is put on particular rice stem borer species in Southeast Asia based on their ecological relevance, impact on the economy, and functional significance.

In many literature reviews, *Scirpophaga incertulas*, *Scirpophaga innotata*, *Chilo suppressalis*, *Chilo polychrysus*, and *Sesamia inferens* were found to be important stem borer species around Southeast Asia regions. The focus of this review is on the respective species.

The species selection was done according to their feeding behaviour, the time when they penetrate the paddy stem, and their interaction with rice phenology. These factors may indicate the rate of damage irreversibility and provide a management regime for the farmers. With this approach, this review can remain aligned with relevant management processes instead of geographically biased reporting intensity. Across the literature review, there are many findings on stem borer species having distinct feeding behaviour, as well as their preferred location within the rice stem, and their own synchronisation with rice growth stages. These suggest a crucial effect of how damage can be different according to their species under field conditions, especially for places that possess multiple prevalent stem borer species. Understanding their distinctive infestation patterns helps us to interpret how different the damage done by each species could be, and the corresponding interaction and effect on rice phenology.

Despite extensive review on this pest, much of the literature often discusses the abundance of the pest, direct yield loss and management, often treating this pest as a similar internal feeder. There is less on species-specific feeding behaviour, oviposition, life cycle, and their corresponding relation to rice phenology. This review aims to fill in the gap by correlating the relationship of the stem borer life cycle, feeding, and oviposition behaviour with rice phenology to produce a better stage-specific management in Southeast Asian regions' rice systems.

REVIEW METHODOLOGY

In this narrative review, existing literature on rice stem borers was synthesised, with a focus only on Southeast Asian countries. Google Scholar, Scopus, and Web of Science were used as scientific databases to perform a thorough literature search. A combination of keywords such as "*rice stem borer*", "*paddy pests*", "*stem borer feeding behaviour*", "*life cycle*", "*oviposition of stem borer*", "*Southeast Asia*", and "*rice insect pests*" was used to identify relevant publications, and the selected publications were chosen from the years between 2000 and 2025. To refine the search results in current databases, Boolean operators such as AND and OR were also used. In this review, a few selected grey literature sources were cross-checked and used to provide regional production. The sources were not used to support a mechanistic interpretation of stem borer biology or any management regime.

Studies on major rice stem borer species affecting paddy fields, their life cycle, feeding pattern, and oviposition behaviour, their corresponding damage characteristics, and relevant relationships to rice production systems in Southeast Asia were included.

This is also put as inclusion and exclusion criteria set for studies in tables. Studies that are not at the species-level resolution or phenology-linked to paddy may be discussed narratively in this review. Distinctions are also made between controlled experimental studies and farmer field observations, where possible, especially when interpreting yield loss, compensation capacity, and effectiveness of management systems in real field conditions.

Rice Cultivation and Production

To provide a regional context for these dynamics, Table 1 summarises rice production, consumption, and trade characteristics of Southeast Asian countries. This contextualization highlights the economic and food security relevance of stem borer damage, particularly in countries with high reliance on domestic rice production or limited capacity to compensate for yield losses through imports.

Table 1
Countries in Southeast Asian regions, highlighting rice-producing and planting countries

Countries	Rice Production	Domestic Consumption	Rice Imports	Rice Exports
Malaysia	Approximately 1.44 million tons (Siddharta, 2024), average yield of around 3.0 tons per hectare.	Approximately 2.7 million metric tonnes in 2023, where national production meets 67% of national demand (Dorairaj & Govender, 2023).	Relies on rice imports, 30 - 40% from neighbouring countries, Vietnam, Thailand, Pakistan, and India.	Primarily a net importer but still exports $48 million in 2023 to several countries (Mat, 2023; World Bank, 2024)
Brunei Darussalam	4,200 tons of rice in 2022 (Statista, 2024), average yield of around 1.7 tons per hectare (Galawat & Yabe, 2012).	Information not found in the current literature.	Imports approximately 28.324 million BND (Brunei Dollars) worth of rice from outer countries.	Relies heavily on imports but exports an amount of 0.002% of total merchandise exports.
Cambodia	Produced approximately 11.62 million tons of rice, with 3.29 tons per hectare. (Khmer Times, 2023; USDA Foreign Agricultural Service, 2024).	142 kilograms per year for per capita consumption (USDA Foreign Agricultural Service, 2024).	Imports are relatively low considering its total production per year.	Export approximately 656,323 tonnes of milled rice in the marketing year of 2022/2023 (USDA Foreign Agricultural Service, 2024).

Table 1 (*continued*)

Countries	Rice Production	Domestic Consumption	Rice Imports	Rice Exports
Indonesia	Rice production is projected to be 30.34 million in 2024.	Domestic rice consumption is projected to be approximately 31 million tonnes.	Indonesia's rice imports have significantly increased, often exceeding 3 million tonnes annually.	Relatively low, it focuses more on ensuring a sufficient domestic supply.
Laos (Lao People's Democratic Republic)	Projected to produce approximately 1.7 million metric tons of rice.	Estimated to be around 1.8 million metric tons.	Imports about 100,000 metric tons of rice, primarily glutinous rice from Vietnam and Thailand.	Forecasted at approximately 80,000 metric tons.
Philippines	Expected to produce around 12.5 million metric tons of rice in 2024.	Projected at about 13.5 million metric tons.	Importing around 1.5 million metric tons annually.	Exports minimal amounts of rice, generally less than 100,000 metric tons.
Myanmar	Estimated at around 13.5 million metric tons for 2024.	Projected to be approximately 12 million metric tons.	Imports about 100,000 metric tons of rice, mainly due to quality preferences and specific market demands.	Exports around 2 million metric tons, primarily to neighbouring countries like China and Bangladesh.
Thailand	Expected to be about 20 million metric tons in 2024.	Projected at approximately 10 million metric tons.	Imports very little rice, typically less than 50,000 metric tons, as it is a major exporter.	One of the largest exporters globally, with expected exports of around 7 million metric tons.
Vietnam	Approximately 43 million metric tons.	Projected at around 30 million metric tons.	Imports a small amount of rice (around 50,000 metric tons).	Exports approximately 6 million metric tons, making it one of the top rice exporters globally.
Singapore	Has negligible rice production due to limited agricultural land; local production is below 10,000 metric tons.	Estimated at about 600,000 metric tons.	Imports virtually all its rice needs, sourcing from countries like Thailand and Vietnam, with imports around 600,000 metric tons annually.	Does not export significant amounts of rice due to its reliance on imports.

Note. Country-level production data are presented for contextual purposes and do not influence the biological interpretations or conclusions drawn in this review

Yield Loss and Economic Impacts of Rice Stem Borers

Rice stem borers, as described, are among the most destructive rice pests around the world, causing significant economic loss. According to Subedi et al. (2024), they described the borer as one of the most critical insect pest groups, highlighting the need to be prioritised in management control due to the severity and consistency of damage they cause to rice crops. The expected damage is caused by the destructive larval stage, where the larvae bore and feed inside the stem, which later produces two common symptoms: deadheart (during the vegetative stage) and whitehead (during the reproductive stage), as shown in Figure 1. Both symptoms affect the production of grain yield, leading to a critical decline in production when at high infestation levels.

The impact on yield production can be different due to distinct infestation timing related to rice growing stages. Deadheart symptoms that occur during the vegetative phase may be partially compensated through the production of new paddy tillers. Meanwhile, the later stage of deadheart, which is whitehead, occurs after panicle initiation, where the larva keeps boring throughout the stem, eventually disrupting the translocation of nutrients to vascular tissue supplying the developing panicle. At a later stage, the respective plant has limited capacity for compensatory growth as compared to during the vegetative stage, which translates directly into irreversible yield loss. This may explain why relatively low levels of whitehead incidence often result in higher large reductions in grain yield compared with deadheart damage.

Figure 1. Observed damage symptoms caused by rice stem borers at different stages: (a) Deadheart at the paddy vegetative stage, showing gradual death of the central tiller due to larval feeding (surrounding tillers remain green); (b) Whitehead at the paddy reproductive stage, shown by creamy white, empty panicles

These differences are clearly reflected in long-term yield-damage relationships. Based on an analysis of 770 experimental units conducted over 28 years (1965-1992), Muralidharan and Pasalu (2006) estimated the effects of rice stem borer injury on grain yield across major rice ecosystems in Asia. In irrigated systems, a 1% increase in deadheart incidence resulted in only a 0.3% reduction in yield (approximately 12 kg ha^{-1}), whereas a 1% increase in whitehead damage caused a substantially higher yield loss of 4.2% (approximately 183 kg ha^{-1}). In rainfed lowland systems, where plants exhibit greater capacity for compensatory tillering, both 1% deadheart and whitehead damage were associated with an approximate yield reduction of 2.3% (about 76 kg ha^{-1}). The much greater impact of whitehead damage reflects its direct association with the loss of productive panicles, in contrast to the partially recoverable effects of deadheart damage. Recent studies also supported the idea, showing that the relationship between stem borer injury and yield loss is not fixed but depends on several factors. For instance, rice variety, growth stage, and environmental conditions may influence how much damage translates into yield losses (Horgan et al., 2021).

Between irrigated and rainfed systems, recent reviews emphasise there is no fixed threshold between both systems, but they do depend strongly on the system itself, varietal tolerance, and environmental conditions (Khan et al., 2020; Sapkota et al., 2019). Zhao et al. (2018) and Nguyen et al. (2022) mentioned in a physiological context that irrigated rice exhibits stronger compensatory growth due to wider availability of nutrients and water, as compared to the rainfed system, where the compensatory growth may be limited due to a smaller source of water and nutrients.

Reported yield losses caused by rice stem borers vary widely across studies (Savary et al., 2000; Litsinger et al., 2011). Severe, localised outbreaks can result in losses as high as 70% (Rahim et al., 1992). There is no direct relationship between larval density and reduction in yield. Yield reduction may be heavily influenced by different factors such as crop growth stage, compensation ability, and planting density (Villegas et al., 2021). Previous literature studies commonly described the association of yield loss with damage symptoms (deadheart and whitehead incidence) as compared to larval counts. In Southeast Asia, yield losses attributed to rice insect pests, stem borers, gall midges, and planthoppers range from 21% to 50% (Pasalu et al., 2004).

Also, most published literature on the relationship between stem borer damage and yield loss often describes using older rice varieties and management practices. Hence, the described loss estimate is more recommendably viewed as a general risk indicator than a precise estimate for modern high-yielding cultivars grown under intensified systems nowadays. The overall damage estimate patterns can still be used by researchers and farmers to understand the occurrence of damage, and so the stages of paddy are the most vulnerable.

Since the infestation of stem borers is unpredictable, many farmers continue to manage the issue using chemical pesticides. Insecticide sales have increased to USD 674 million (2012) from USD 409 million (2009) in South and Southeast Asia. Cabasan et al. (2019) described 62% of the farmers utilise pesticides to control stem borers and leaf folders.

This is supported by Mondal et al. (2017), where pesticide use represents the second-highest in production cost after plant nutrition. This highlights the concern caused by stem borers and calls the need for more sustainable and stage-specific management control.

Ecology and Distribution of Rice Stem Borers in Southeast Asia

Rice stem borers are described as a type of lepidopteran pest, with the most identified species being *S. incertulas*, *S. innotata*, *C. suppressalis*, *C. auricilius*, and *S. inferens* in Southeast Asian regions. In these regions, the climate is often characterised by high temperatures and humidity. Continuous or staggered planting cycles promote ideal conditions for the survival of stem borers and also produce multiple generations throughout the year. Despite this, differences in crop intensity, irrigation regimes, landscape structure, and planting synchrony may influence the prevalence of species and abundance in the area.

The diversity and species distribution prevalent in an area are crucial to be determined due to their difference in biological traits and preferences, as this will in the future be considered in management strategies, from monitoring, determination of threshold level, to deciding which control measures are the most appropriate. In Table 2, the major rice stem borer species reported across Southeast Asian countries are highlighted.

Table 2
Major stem borer species in Southeast Asian countries

Countries	Rice Stem Borer	References
Cambodia	*C. suppressalis, S. incertulas*	Dunn et al. (2023); Castilla et al.(2020)
Indonesia	*C. suppressalis, S. innotata*	Agustina & Herlina (2025); Wijarya et al., (2024); Subba et al. (1969)
Laos (Lao People's Democratic Republic)	*C. suppressalis, S. incertulas*	Coulibaly et al. (2021); Douangboupha et al. (2006)
Malaysia	*C. polychrysus, S. incertulas, S. innotata, C. auricilius, C. suppressalis, S. inferens*	Mohd Khari & Ab Hamid (2022); Ling et al. (2020); Yeo et al. (2025); Gumbek & Hamsein (2011)
Philippines	*S. incertulas, C. suppressalis, S. innotata, S. inferens, C. auricilius*	Viz & Pacada (2024); Horgan et al. (2021); Litsinger et al. (2011)
Myanmar	*S. incertulas*	Maung et al. (2020); Pham & Kwon (2024)
Thailand	S. incertulas	Arunmit et al. (2024); Catling et al.(1984)
Vietnam	*S. incertulas, C. suppressalis*	Quang et al. (2025); Pham & Kwon (2024); Thuy et al. (2022)
Brunei Darussalam	Published documentation on rice stem borer incidence in Brunei Darussalam remains limited, with few peer-reviewed studies specifically addressing species distribution or damage severity.	
Singapore	Limited rice cultivation, hence fewer literature sources regarding borer species.	

Note. S. incertulas (Yellow stem borer); *S. innotata* (Rice white stem borer); *C. auricilius* (White-backed rice borer); *C. polychrysus* (Dark-headed rice borer); *C. suppressalis* (Striped rice stem borer); *S. inferens* (Pink stalk borer)

Life Cycle, Developmental Stage and Feeding Behaviour

Rice stem borer is a lepidopteran and holometabolous insect which complete their life cycle through four different developmental stages. It starts from adult moths laying eggs on rice plants. The eggs will hatch into larvae that will undergo several larval instars, which vary among species. The larval stage, known as the most damaging stage to rice crops, is where they bore and feed within rice stems before entering the pupal stage (Figure 2). Pupation occurs where they embed themselves within protective cocoons, formed inside the stem or plant debris, after which they will emerge into adult moths in a few weeks to complete the life cycle.

Looking closer at how different species feed, *Scirpophaga* species usually feed inside the central pith of a single tiller and are less mobile, creating more localised damage and a quicker appearance of damage symptoms. *Chilo* species are more mobile, where their larvae move between tillers, making the damage more spread out and develop more gradually, making early symptoms more difficult to notice in the field. Compared to both *Scirpophaga* and *Chilo*, *Sesamia* species show more flexible feeding behaviour. For instance, *S. inferens* larvae often start by feeding on leaf sheaths before boring into the stem. Their ability to develop on rice as well as other grasses suggests they may infest different plant tissues at various growth stages (Li et al., 2023; Sau et al., 2022).

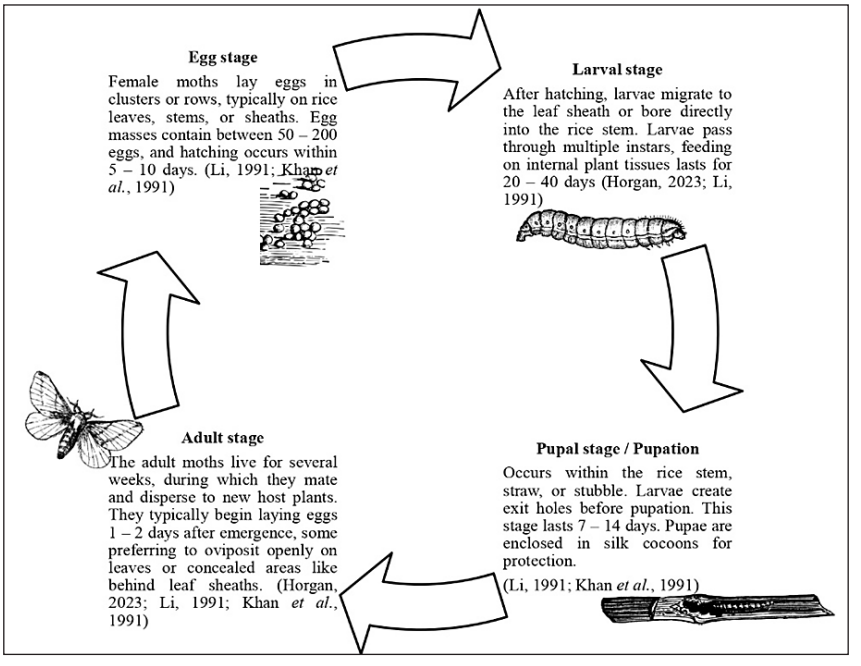

Figure 2. The general life cycle of the rice stem borer consists of four distinct stages: egg, larva, pupa, and adult. The estimated total life cycle is approximately 34 to 55 days

*Source: Adapted from Horgan (2023) and Li (1991)

Evidence for direct interactions among rice stem borer species remains limited. Most studies suggest that different species can coexist because they exploit the rice plant in different ways, rather than through strong competition. Variations in oviposition timing, larval feeding position within the stem, and preference for rice growth stages help reduce overlap within the same tillers.

When multiple stem borer species occur together, damage severity is more likely determined by combined feeding pressure and synchronisation with susceptible rice growth stages than by direct interactions among species (Table 3 and Table 4). Understanding these indirect relationships helps explain variable damage outcomes in multi-species infestations. Under field conditions where multiple species coexist, yield loss is therefore more strongly influenced by cumulative internal feeding across growth stages than by direct competition.

Linking Species-specific Behaviour to Rice Phenology

Linking species-specific behaviour to rice growth stages helps the researchers explain how damage develops in the field. Damage caused by stem borer depends not only on when the pest is present, but also on how each species feeds and where it attacks the plant. Differences in egg-laying behaviour and larval feeding patterns determine when, where, and how severe the damage becomes (Table 5).

Damage expression in rice stem borers is closely linked to the synchronisation between pest development and crop phenology. However, interspecific variation in oviposition behaviour and larval feeding mechanisms (Tables 3 and 4) governs the timing, location, and severity of injury across growth stages.

Early Vegetative Stage: Different Establishment Success

During the early vegetative state, there are differences in how larvae establish themselves within the young rice plant. For *Scirpophaga* species, their neonates quickly move

Table 3
General comparison of feeding patterns between Chilo spp., Scirpophaga spp., *and* Sesamia spp.

Feature	*Chilo* spp.	*Scirpophaga* spp.	*Sesamia* spp.
Early Feeding	Leaf sheath/whorl	Minimal external feeding	Briefly on the leaf sheath
Entry into Stem	Early-mid larval stage	Very early	Early after short sheath feeding
Feeding Depth	Stem internodes	Central stem or growing point	Deep stem tunnelling
Host Range	Moderate	Mostly the rice plant host	Broad (rice, maize, sorghum)
Damage Pattern	Deadheart, Whitehead	Severe deadheart, whitehead	Deadheart, hollow stem, whitehead

Table 4
Detailed comparison of life cycle, oviposition behaviour, and feeding mechanism of major rice stem borers in Southeast Asia

Features	*Chilo* spp.	*Scirpophaga* spp.	*Sesamia* spp.
Life cycle			
	• Larval development comprises up to 7 instars and typically lasts approximately 20-30 days, depending on temperature and host condition. • Pupation occurs within the rice stem or between the leaf sheath and stem tissues. • Adult emergence is temperature-dependent, generally occurring above 15-16ºC. • In tropical regions, multiple generations may occur annually, whereas two generations are typical in temperate zones.	• Eggs hatch within five to eight days under favourable conditions. • Larvae feed internally for approximately 20-35 days. • Pupation occurs inside the stem, usually near the plant base. • Adult lives for approximately three to five days. • Three to five generations per year may occur, depending on climatic conditions.	• Larvae undergo six to eight instars, with development lasting approximately 30-70 days depending on host plant and season. • Pupation occurs inside the stem or near the plant base and lasts 9-11 days. • Adult longevity ranges from five to seven days. • Four to five overlapping generations may occur annually in tropical environments.
Oviposition Behaviour			
Location of Egg-laying	Eggs are laid in flattened, fish-scale-like masses on the underside of leaves, typically near the leaf sheath and plant base.	Eggs are deposited in compact masses covered with yellowish-brown hairs or scales from the female abdomen, usually on the upper leaf surface near the midrib.	Eggs are laid inside or near leaf sheaths and are rarely deposited on exposed leaf surfaces.
Number of Eggs	Approximately 200 - 700 eggs per female over its lifetime.	Several hundred eggs per female.	Approximately 150-400 eggs per female.
Timing of Oviposition/ Hatching	Mostly at night, with a peak shortly after mating.	Often in a short time window and hatch synchronously within five to eight days.	Duration of seven days, with a peak on the second day after adult emergence.
Feeding Mechanism			
Chilo spp.	Newly hatched larvae remain clustered within the leaf sheath before stem penetration. Early feeding causes sheath withering and superficial lesions. Larvae bore into the stem typically by the third instar (approximately 10-12 days after hatching) and remain concealed thereafter. Feeding primarily occurs within stem internodes rather than exclusively at the central growing point.		

Table 4 (*continued*)

Feeding Mechanism	
Scirpophaga spp.	Neonate larvae rapidly migrate downward after hatching. They penetrate the stem directly through the leaf sheath and bore toward the central growing point. Early feeding is concentrated at the apical meristem, leading to rapid expression of deadheart symptoms.
Sesamia spp.	Neonate larvae initially remain within or behind the leaf sheath. Early feeding produces water-soaked or gummy lesions. Larvae subsequently bore into the central stem and may tunnel across multiple internodes (first to fifth internodes). Later instars may exit and re-enter adjacent tillers, increasing cumulative plant damage.

Table 5
The synchronisation of stem borer activity with critical phases of rice growth

Species	**Feeding Focus**	**Dominant Symptom Stage**	**Compensation Potential**	**Risk Window**
Scirpophaga spp.	Apical meristem	Early deadheart	Lower recovery	Early vegetative
Chilo spp.	Internodes	Progressive vascular damage	Moderate recovery	Vegetative to booting stage
Sesamia spp.	Multi-internode + movement between tiller	Cumulative late damage	Low recovery	Booting to the grain filling stage

downward after hatching and feed directly at the growing point, leading to the swift appearance of deadheart formation. In comparison, *Chilo* larvae initially remain within the leaf sheath and only later bore into the stem, usually around the third instar. Their feeding often occurs within the stem internodes rather than directly at the meristem. *Sesamia* larvae may remain within or behind the sheath initially, and later instars can exit and re-enter adjacent tillers, increasing overall plant damage.

These differences affect how damage appears in the field. Because *Scirpophaga* targets the growing point early, deadheart symptoms tend to appear earlier and more suddenly compared to other species. *Chilo*, with a slight delay in stem penetration, usually causes more gradual symptom development. Meanwhile, *Sesamia* can create more scattered damage due to its movement between tillers. As a result, even when infestation levels are similar, the severity and pattern of damage at early stages can vary between species.

Differences between early- and late-planted rice can modify larval establishment and feeding intensity; field studies consistently report lower stem borer incidence in early-planted crops and elevated infestation in late-sown crops (Patil & Rao, 2020). Although direct movement tracking is limited, repeated field observations indicate that staggered planting creates continuous host availability that facilitates inter-field movement and increased pest pressure in later plantings (Heong et al., 2010).

Tillering Stage: Interaction with Compensatory Capacity

During the active tillering stage, larval tunnelling disrupts the vascular system of the plant and interferes with nutrient movement. However, the level of damage and the ability of the plant to recover depend on where different species feed. Since *Scirpophaga* usually feed at the growing point, it may lead to a direct impact on the central tiller and limit further tiller development. *Chilo* species tend to feed within the internodes, which may initially spare the growing point, unlike *Scirpophaga*, gradually weakening the plant's structure and function eventually. While for *Sesamia* species, more widespread disruption may occur due to its mobility tunnelling through several internodes and between nearby tillers.

These differences in feeding behaviour suggest that the mix of prevalent species present in a field may influence both the severity of damage and the plant's ability to recover. Compensatory tillering is most effective when infestation occurs early, and damage is limited to a small proportion of tillers. However, species that concentrate feeding at the meristem may reduce recovery potential compared to species that initially feed within internodes. This suggests that species composition within a field may influence the effectiveness of compensation during vegetative stages.

Reproductive Stage: Whitehead Formation and Yield Impact

At panicle initiation and heading stages, stem borer infestation results in more severe consequences, as all species ultimately disrupt the vascular system of the plant that is necessary for nutrient translocation for panicle development and grain filling. However, differences in tunnelling behaviour may influence the timing and expression of symptoms. From the initial tillering stage, *Scirpophaga* neonate's early infestation on the apical meristem may induce whitehead formation shortly after the paddy booting stage. *Chilo* neonates that feed across stem internodes may cause whitehead symptoms when vascular tissues supplying the developing panicle are sufficient. *Sesamia* neonates are capable of tunnelling across multiple internodes and occasionally moving between adjacent tillers, which may generate more extensive cumulative disruption during late reproductive stages. Unlike vegetative-stage damage, injury during panicle initiation and heading may be irreversible, as the plant's capacity for compensatory tillering is minimal at this stage. Consequently, species that sustain feeding during reproductive development may contribute disproportionately to final yield loss.

Control Management of Rice Stem Borer

In history, recent syntheses highlight the role of insectivorous birds and bats in suppressing arthropod pests in rice landscapes, supporting conservation of bird habitat as a complementary pest-suppression strategy (Gogoi, 2023; Sottomayor, 2024).

The pests are also primarily controlled through cultural practices, including altering transplanting dates, flooding fallow fields, manually removing egg masses, and digging or burning stubble, in addition to using light traps (Luo et al., 2021). Over the years, the management has been improved to manage these pests based on several methods, including biological method, biochemical method, microbial method, chemical method and varietal resistance.

Biological Control Method

In Southeast Asian countries, biological methods are getting more attention as their various approaches within biological means to manage pests (Babendreier et al., 2022). One of the most common biological control methods is using natural enemies. Natural enemies, including egg parasitoids, are extensively utilised and studied. For instance, *Trichogramma japonicum* is highly deployed as a biological control agent in many regions. They are highly utilised for their high feasibility to mass rear and target major rice stem borer species, especially *S. incertulas* and several *Chilo* species. There are many field releases of *T. japonicum* in the Southeast Asian regions, including Indonesia, Philippines, Vietnam, Malaysia, and Thailand, commonly reporting moderate to high with a satisfactory rate of egg parasitism under optimal conditions (Horgan 2023; Subedi et al., 2024). *T. japonicum*, in the form of small parasitic wasps, commonly oviposit within host eggs that will prevent larval emergence and thus suppress crop damage at an early stage. This is highly favoured in IPM programmes due to their high specificity, environmental friendliness, and good compatibility with ecological pest regulation (Pasalu et al., 2004).

Apart from T. japonicum, there are more parasitoids used as biological control of stem borers in Southeast Asia, as presented in Table 6. Other egg parasitoids such as *Telenomus rowani*, *Telenomus dignus*, and *Trichogrammatoidea nana* have been found to be useful as biological control, with reported field parasitism of 10 - 60% (depending on habitat conditions and management regimes). *T. rowani* is reported to exhibit more stable parasitism under less to non-disturbed field conditions (low-input or unsprayed systems), whereas *Trichogramma* performance is more habitat-dependent and sensitive to insecticide exposure.

In contrast, pupal parasitoids such as *Tetrastichus schoenobii* are also reported to achieve localised parasitism levels of up to approximately 40%, yet their effectiveness is strongly affected by insecticide regimes and seasonal host availability. Generally, parasitoids, including *Cotesia flavipes* and related braconids, are present but generally play a secondary role in rice ecosystems because stem borer larvae remain concealed within plant tissues, limiting parasitoid access and reducing consistent field impact.

Despite reports of moderate to high parasitism, these effects are frequently inconsistent across seasons and locations. High parasitism does not necessarily mean stable stem borer suppression or yield protection, largely due to poor synchrony between persistent parasitoid

activity and stem borer oviposition, intensive insecticide use, and simplified rice landscapes that limit parasitoidal interactions.

Recent evidence further suggests that habitat complexity, crop mosaics, and planting synchrony help to increase parasitoid effectiveness by influencing both pest movement and the availability of host stages (Dominik et al., 2018; Saksongmuang et al., 2024). Diversified and ecologically managed rice systems may enhance parasitism; these benefits are often context-dependent, which may be affected by many external factors rather than consistent across all environments. Overall, evidence from egg, larval, and pupal parasitoids suggests that the success of biological control in Southeast Asian rice systems depends more on factors such as crop phenology, landscape structure, and management practices. This may suggest the need for biological control strategies that are aligned with crop growth stages and supported by favourable habitat conditions (Catalayud et al., 2006).

Collectively, the synthesis of field parasitism patterns across egg, larval, and pupal parasitoids indicates that parasitoid efficacy in Southeast Asian rice systems is governed less by intrinsic biological potential and more by crop phenology, landscape structure, and management intensity, underscoring the need for phenology-aligned and habitat-supported biological control strategies (Calatayud et al., 2006).

Table 6
Major parasitoids targeted at the stem borer

Parasitoid	Target stage	Countries	Analysis
Trichogramma japonicum	Egg	Indonesia, Philippines, Vietnam, Malaysia, Thailand	Most widely released, however, performance is strongly habitat-dependent
Trichogrammatoidea nana	Egg	Indonesia, Malaysia, Thailand, Vietnam	Effective egg parasitoid under augmentative release
Telenomus rowani	Egg	Indonesia, Malaysia, Philippines	Often more stable than Trichogramma under farmer fields
Telenomus dignus	Egg	Philippines, Indonesia, Malaysia	Native egg parasitoid contributing to natural biological control in unsprayed fields
Eriborus sinicus	Larva	Philippines, Vietnam	Important larval parasitoid of *Chilo* spp., the effectiveness varies with host density and insecticide exposure
Cotesia flavipes	Larva	Indonesia, Philippines, Vietnam, Thailand	Key larval parasitoid of stem borers; widely used in classical biological control and IPM programmes
Elasmus albopictus	Larva	Philippines, Indonesia	Secondary parasitoid, population strongly influenced by cropping system and pesticide use
Tetrastichus schoenobii	Pupa	Indonesia, Philippines	Sensitive to insecticide regimes

Biochemical Control Method

The use of semiochemicals is another biological method for controlling stem borers, as these compounds are naturally released by plants and animals. This category includes sex pheromones, which are widely utilised to attract and trap insect pests (mass trapping), implement 'lure and kill' strategies, and disrupt mating processes. According to Regnault-Roger (2012) and El-Sayed et al. (2006), the diffusion of artificial pheromones can effectively interfere with the natural pheromone communication between male and female pests, reducing their ability to mate.

In Southeast Asian rice systems, pheromone-based control (Z)-11-hexadecenal (± Z9-16:Ald, Z13-18:Ald) is mainly used to manage lepidopteran stem borers. These species rely on sex pheromones to find their mates, which makes them a suitable target for this method. In general, three main deployment strategies are commonly used. First, low trap densities (about 1-2 traps per hectare) are applied to monitor pest flight activity and seasonal patterns. Second, moderate to higher trap densities are used for mass trapping to reduce the male populations, and lastly, by conducting area-wide mating disruption strategies that aim to saturate the field with pheromones so the males cannot locate the females. The third approach is becoming more important in rice systems because stem borer larvae remain hidden inside plant tissues and are difficult to control directly (Vacas et al., 2016).

The recommendations of using around 20 traps per hectare, with each containing about 5 mg of synthetic pheromone, have been supported by field studies, but still depend on the local conditions. Research shows that the trap densities below about 10 traps per hectare are usually not effective in reducing mating, especially in rice landscapes where pests continuously move between fields. In contrast, about 20 traps per hectare is considered a practical minimum level to ensure enough overlap of pheromone signals to interfere with the male orientation. This setup requires roughly 100 mg pheromone per hectare and matches regional IPM guidelines, which emphasise that the total pheromone amount per area is more important than trap number alone.

However, pheromone-based control does not always work the same way under different pest densities. When pest populations are low to moderate, mass trapping can delay mating and reduce egg laying. But under higher pest density, which is common in Southeast Asian rice fields, mass trapping alone is often less effective. This is mainly due to trap saturation, strong competition from naturally calling females, continuous pest movement from neighbouring fields, and the short mating period of stem borers. Consequently, pheromone-based suppression under high pest pressure is more reliably achieved through mating disruption integrated within additional and broader IPM strategies rather than through mass trapping alone (Balingbing et al., 2025; Hajjar et al., 2023).

The use of botanical extracts has also become a common and practical biological approach to managing rice stem borers. These methods are considered safer alternatives compared to conventional pesticides, especially when applied in specific target areas.

Studies across Southeast Asia show that semiochemicals and plant-based extracts mainly work due to their influence on insect behaviour and support for ecological balance instead of killing the pests directly. For example, volatiles released from host and non-host plants can attract or repel insects and are often used in push-pull strategies to manage the movement of stem borers in rice fields. Plant defence activators such as jasmonic acid, salicylic acid, and methyl salicylate can further support pest management by stimulating the release of herbivore-induced plant volatiles. These signals will attract natural enemies, particularly *Trichogramma* spp., which play an important role in controlling the populations of stem borer. Among the botanical products tested, citrus-derived orange oil and neem-based formulations have shown the most consistent performance under field conditions, helping to reduce the damage from stem borer while still conserving the beneficial insects.

Overall, these approaches highlight the gradual shift in the IPM of Southeast Asian rice from relying heavily on single chemical inputs toward more integrated strategies. Farmers are increasingly combining pheromone-based techniques, plant-derived repellents, and natural biological control to achieve more stable and sustainable stem borer management (Table 7).

Table 7
Semiochemicals and plant-derived extracts used for rice stem borer management in Southeast Asia

Category	Compound/Extract	Target Stem Borer	Mode of Action	Sources
Host Plant Volatiles	Hexanal, nonanal, benzaldehyde, decanal	*S. incertulas*	Host attraction, oviposition stimulation	Adak et al., (2024)
Non-host/ Repellent Volatiles	2-octenal, 2-pentylfuran, menthol	*S. incertulas*	Repellence, oviposition deterrence	Adak et al., (2024)
Plant Defence Elicitors (HIPVs)	Jasmonic acid (JA), salicylic acid (SA), methyl salicylate (MeSA)	Rice stem borers	Induces plant volatiles; indirect control	Rani & Murali-Baskaran (2026)
Botanical Extract				
Citrus	Orange oil (Citrus spp.)	Rice stem borers	Repellent + neurodisruptive	Chou et al., (2022)
Neem	Neem oil/azadirachtin (*Azadirachta indica*)	Rice stem borers	Antifeedant, growth regulator	Majlish et al., (2015)
Legumes	Karanja (*Pongamia pinnata*), mahogany oils	Rice stem borers	Repellent, feeding inhibition	Majlish et al., (2015)
Roots/Leaves	*Derris elliptica*, *Neorautanenia mitis*, tobacco, *Adhatoda*	*Chilo* spp., *Scirpophaga* spp.	Toxicity and repellence	Dutta et al., (2024)
Trap Plant/ Plant-derived Semiochemicals	Vetiver (*Vetiveria zizanioides*) terpenes	*C. suppressalis*	Attraction (trap plant)	Li et al., (2025)

Microbial Control Method

Microbial control method is also one of the control methods that is gaining attention among farmers and researchers. Widely used microbial control agents such as *Bacillus thuringiensis*, *Beauveria bassiana*, and *Metarhizium anisopliae* are used for managing rice stem borer. As well as a biological control method, this method is also considered safe as it specifically targets insect pests without disrupting the populations of natural enemies.

Conducted screening studies on entomopathogenic fungi by Shahriari et al. (2021) reported that there are indigenous or locally isolated strains of *Beauveria* and *Metarhizium* that possess high virulence and better environmental endurance compared to some commercial strains, highlighting the importance of local adaptation in microbial control regimes. Mechanistic studies, as described by Wang et al. (2021) and Sutarman et al. (2023), found that *B. bassiana* produces beneficial secondary metabolites (beauvericin and bassianolide) that possess the ability to suppress host immune responses and accelerate mortality. These findings support the use of entomopathogenic fungi as slow-acting but persistent agents, particularly suitable for integration into IPM rather than as stand-alone replacements for chemical insecticides.

Apart from entomopathogenic fungi, beneficial bacteria such as *B. thuringiensis* are also widely used against rice stem borer. Recent reviews and genomic studies (2023-2025) demonstrate the continued effectiveness of Cry1Ab/Cry1Ac toxins against rice stem borers, especially when expressed in transgenic rice lines. However, resistance monitoring studies published up to 2025 provide evidence that low-frequency Cry resistance alleles are present in some *C. suppressalis* populations (Hu et al., 2024; Li et al., 2023). For example, in Cambodia, *B. bassiana* and 6% cold-pressed orange oil have been successfully used to manage insect pests in rice fields. In Malaysia, *B. thuringiensis*, as well as *Skermanella* sp. and *Serratia* sp., are also widely used as microbial control at concentrations of 3.80×10^4 CFU ml^{-1} and 2.29×10^5 CFU ml^{-1}, respectively, to mitigate *C. medinalis* and *S. inferens* (Abdullah & Mokhtar, 2024; Panneerselvam et al., 2018)

Importantly, recent field-oriented syntheses indicate that microbial agents perform best when deployed as part of integrated pest management systems, combined with cultural practices, biological agents, and proper chemical inputs. Studies from tropical and subtropical rice systems report that environmental factors, especially humidity, temperature, and canopy structure, strongly influence the persistence and efficacy of entomopathogenic fungi, explaining variability in field outcomes across locations (Jaronski, 2010; Mascarin & Jaronski, 2016). These studies provide insights into the potential of microbial agents for rice stem borer management; however, the transferability of findings beyond the studied regions remains uncertain. While the pest shares broadly similar feeding behaviour and physiological traits across geographic regions, the field-level efficacy of microbial control agents is strongly influenced by environmental conditions, host population dynamics, formulation characteristics, and application practices.

Overall, reported findings from Southeast Asian rice systems on microbial control methods should also be conveyed as indicative rather than predictive for other regions. As well as other natural methods, microbial control also relies heavily on external factors, hence requiring additional region-specific validation trials. Local strain screening and resistance monitoring are recommended to promote local adaptation and to confirm validity before being used in broader applicability.

Chemical Control Method

Chemical pesticides remain an important tool for managing pest and disease challenges in agriculture. The use of synthetic organic, inorganic, and natural chemicals, such as pyrethroids, insect growth regulators, and fumigants, is considered a chemical control method. Their use is guided by Good Agricultural Practices (GAP) to ensure safe, effective, and responsible pesticide selection and application (Table 8). This approach minimises the risks associated with excessive or improper pesticide use, safeguarding the health of farmers, consumers, and the environment.

There are reported findings of high-yielding production under intensive management through extensive use of chemical insecticides. It has been reported by Peng et al. (2000) that rice yield potential in Asia can reach approximately 10 t ha^{-1} when high-input pest control strategies are employed. For instance, long-term evaluations conducted by the International Rice Research Institute, encompassing 117 experiments over fifteen years, demonstrated that the production of insecticide-treated plots produced up to 87% higher yields than untreated plots, with benefit-cost ratios ranging from 3 to 10 (Pathak & Khan, 1994). These findings highlight the substantial contribution of chemical insecticides to yield protection, particularly under high pest pressure.

Several control strategies have been recommended to achieve effective suppression of rice stem borers during vulnerable crop stages. These include prophylactic or early-stage interventions such as seedling root soaking with systemic insecticides, as well as targeted applications of granular formulations during key developmental phases. Such approaches reflect the importance of aligning insecticide use with periods of heightened pest susceptibility.

Table 8
Comparison of the efficacy of chemical pesticides against the stem borer

Effective	Moderately Effective	Highly Effective	Source
Carbaryl	Phosphamidon, quinalphos	Monocrotophos, fipronil, chlorpyrifos, phosalone, isazophos, carbofuran, and fenthion	El-Wakeil et al., (2013); (Seni & Naik, 2017)
Safer from natural enemies	Carbofuran, phorate, cartap, isazophos, triazophos, acephate.		

*Source: Modified from Yadav et al. (2021) and Pasalu et al. (2004)

Despite these demonstrated benefits, prolonged and intensive reliance on chemical insecticides has raised significant ecological and management concerns. Repeated application of broad-spectrum insecticides can disrupt populations of natural enemies, weakening biological regulation within rice ecosystems and increasing the risk of pest resurgence once insecticide residues decline (Ali et al., 2019). Repeated use of similar insecticides may place strong selection pressure on stem borer populations, increasing the risk of pest resistance, especially when applications are not well timed with the pest's life cycle.

The development of rice stem borer has a strong dependent relationship with the effectiveness of chemical control. Application of chemical pesticides when larvae have already hatched from the eggs may not be as effective as they become physically protected by the paddy's external structure. This may result declination in control efficacy and may further accelerate resistance development by exposing the pest to sub-lethal doses. Thus, correct time applications targeting early infestations can help to achieve effective control at a lower input level and simultaneously reduce the frequency of insecticide use.

Thus, judicious use of chemical insecticide is only recommended when infestation has exceeded the established economic threshold, as suggested by Saad Abdullah et al. (2012) that application should be made when approximately 10% of hills are infested or when four egg masses per hill are detected. 10% deadheart incidence is widely cited as a threshold; the application of chemical control should be adjusted to local or regional pest pressure, crop stage, and production system. Generally, applications made after the appearance of whitehead symptoms are ineffective, as the damage to reproductive tissues is irreversible. These highlight the importance of correct timing and threshold-based decision-making in creating control strategies and highlight the need for judicious use of chemical control with IPM approaches to ensure long-term sustainability.

Varietal Resistance

Host plant resistance to rice stem borers has mainly been approached by developing rice varieties with moderate resistance or field tolerance immunity (Chaudhary et al., 1984; Horgan et al., 2021). Early breeding programmes at IRRI reported several varieties that showed lower levels of deadheart and whitehead damage when exposed to stem borer species. Widely cultivated varieties such as IR20, IR36, and IR50 were reported with moderate resistance and shorter crop duration, making them more practical to be cultivated under field conditions in Southeast Asia rice fields. Other varieties, including Ratna, Chandina, and Taitung 16 (T16), have also been identified as relatively resistant in both experimental and field settings (Table 9).

In the Philippines, recent varietal releases, including NSIC Rc 480 and NSIC Rc 27, highlight the importance of early-maturing rice (107 days) with moderate resistance to

Table 9
Rice varieties in Southeast Asia regions with resistance to stem borers

Variety	Key Regions of Use	Crop Duration	Reaction to Stem Borers
IR20	Widespread Asia (Philippines, Vietnam)	Medium	Moderate resistance
IR36	Indonesia, Thailand	Short	Moderate resistance / tolerant
IR50	Philippines, Vietnam	Medium-short	Moderate resistance
Ratna	Regional (e.g., Indonesia)	Medium	Moderate resistance
Chandina	Southeast Asia	Medium	Moderate resistance
Taitung 16 (T16)	IRRI trials (Philippines)	Medium	High resistance in screening
NSIC Rc 480	Philippines	Short (~107 days)	Intermediate reaction
NSIC Rc 27	Philippines (upland)	Short (~107 days)	Moderate resistance (white stem borer)

stem borers (Philippine Rice Research Institute, 2023). Shorter growth duration limits the available time for stem borers to create infestation, especially during the reproductive stage of the paddy crop. Similarly, in Malaysia, no rice variety has been classified as resistant to stem borers, although some local varieties show an association with lower damage to stem borers (Abdullah et al. 2025).

However, resistance in these varieties is still partial and is heavily influenced by environmental conditions, and up to this moment, no widely grown variety provides complete protection against stem borers. This suggests the stem borer damage and yield loss are more strongly influenced by crop duration and timing of exposure; even when larval infestation occurs, tolerant rice varieties may experience less yield loss. This approach should be considered a risk-reduction strategy that helps the farmers stabilise yield, rather than a complete control solution, while ensuring good agronomic performance.

Integrating Stem Borer Ecology with Management Strategies

Given the wide range of stem borer management strategies used across Southeast Asia, a comparative synthesis is needed to better understand how these approaches can work together in a compatible way. Table 10 presents different control measures to vulnerable stages in the pest life cycle, together with corrective actions across the crop growth stages.

Because yield loss becomes irreversible once larvae penetrate stems during reproductive stages, IPM prioritises early-stage suppression. At the adult stage, habitat and synchrony manipulation, including modified planting geometry and landscape-level synchronous planting, reduce oviposition preference and host continuity. Pheromone monitoring supports threshold-based timing, strengthening decision precision.

Interventions may be intensified at the egg stage, where *Trichogramma* and other parasitoids are released, and conservation of the natural enemies increases pre-entry mortality.

Table 10
Ecology-aligned integrated management framework for rice stem borers in Southeast Asia

Stem Borer Stage	Crop Stage Vulnerability	Management Strategy	Functional Role in IPM	Type of Control
Adult (oviposition stage)	Early vegetative	Jajar Legowo planting geometry (Indonesia)	Reduces oviposition preference via canopy modification	Cultural
	Pre-season	Synchronous planting (Malaysia, Laos)	Reduces asynchronous host availability	Cultural
	Pre-season/all stages	Pheromone monitoring and ETL-based targeted trapping (Vietnam)	Early detection and improved spray timing	Semiochemical/ Monitoring
Egg stage	Early vegetative	Trichogramma release (Malaysia, Vietnam)	Egg parasitism before larval emergence	Biological
	Early vegetative	Conservation of natural enemies, community-based mass release of Trichogramma (Philippines)	Maintains ecological mortality factors	Biological
Early larval stage (before deep stem entry)	Vegetative (tillering)	Alternate wetting and drying (AWD) (Thailand, Vietnam, Philippines)	Disrupts larval establishment	Cultural
	Vegetative	Split nitrogen application (Malaysia)	Reduces excessive vegetative attractiveness	Cultural (Nutrient management)
	Early infestation	Chlorantraniliprole, fipronil (ETL-based use) (Myanmar, Malaysia)	Rapid suppression before irreversible stem damage	Chemical
Late larval stage (internal feeding)	Tillering-booting	Incorporate *B. bassiana*, *M. anisopliae*, and *B. thuringiensis* (Thailand, Cambodia)	Biological mortality of concealed feeders	Biological (Microbial)
	Booting-heading	Targeted insecticide application (ETL-guided)	Prevents whitehead formation during the reproductive stage	Chemical
Overwintering/ stubble survival	Post-harvest	Stubble destruction/crop sanitation	Reduces carry-over population	Mechanical/ Cultural
		Flooding fallow fields	Suppresses diapausing larvae/ pupae	Cultural

During early larval establishment, cultural practices such as alternate wetting and drying and split nitrogen management reduce habitat suitability and plant susceptibility. These measures collectively lower larval survival before structural damage occurs.

Selective insecticides are reserved for threshold-exceeding infestations and function as a corrective safeguard rather than a routine measure. By concentrating chemical inputs at critical risk points, the framework reconciles yield protection with environmental risk reduction, demonstrating that ecological alignment enhances both sustainability and strategic efficiency.

FUTURE TREND AND RESEARCH GAP

In this review, the control management regime could be properly strategised by understanding how the stem borer damage and rice phenology are correlated. However, there are several research gaps that remain unfilled. One of the main research gaps is an experimental study that shows a clear quantification of yield loss by different stem borer species at different paddy growth stages. Since a multi-species complex is quite common in rice fields, to determine yield loss under such condition is necessary to observe the interaction or even dominance of species towards affecting the production of grain yield.

At the current time, a lot of management and control recommendations often treat stem borers as if they behave similarly, though they behave differently in terms of feeding pattern, oviposition behaviour, and life cycle. All these characteristics have effects on the ability of the rice crop to recover from the damage after infestation. While the improvement in management controls by integrating biological, microbial, chemical, and biochemical in a system is applauded, they are still mostly discussed conceptually rather than field-based experimental evidence. Hence, another research gap that may be considered in the future is to produce a management control regime that is species-specific towards rice stem borers at targeted rice growth stages.

Thus, the discussed concepts should therefore be studied in a controlled experiment involving both single and mixed species across targeted rice growth stages. This will help to quantify and provide knowledge for the researchers and farmers to determine the extent of irreversible yield loss, plant compensation, as well as the effectiveness and specificity of each management control towards rice stem borer. By filling in these gaps, the researchers can help to develop more precise, species-specific, and aligned phenology solutions that may improve yield protection while reducing judicious use of chemicals and their detrimental impacts.

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Effect of Boron Fertilisation and Trisodium Phosphate (TSP) Priming on Fruit and Seed Quality of GM-2 Tomato (*Solanum lycopersicum* L.)

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ABSTRACT

GM-2 is a superior tomato accession originating from Indonesia and exhibiting nematode resistance. A problem with GM-2 tomatoes is poor seed quality. Improvements to fruit and seed quality are needed through boron fertilisation and seed priming with Trisodium Phosphate (TSP). The objectives were to evaluate the effect of boron fertilisation on fruit quality and to assess the combined effect of boron fertilisation and TSP priming on seed quality and seedling growth in GM-2 tomato. The study on boron fertilisation was carried out in Ngablak District, Magelang Regency, Indonesia, from October to December 2023, using a CRD with three replications. Boron fertilisation consisted of 0 and 1.25 g.plant^{-1}. The study on TSP priming was conducted at the Faculty of Agriculture, Universitas Gadjah Mada, from December 2023 to January 2024, using a RCBD factorial with three blocks as replications. TSP concentrations ranged from 0, 5, 10, and 15 g.L^{-1}. The results showed that boron fertilisation significantly increased fruit number and fruit fresh weight by 9.38% and 14.34%, respectively, and improved fruit firmness (5.64%) and seed moisture content (1.02%). TSP concentration significantly influenced seedling growth. The TSP concentration of 15 g.L^{-1} increased root surface area by 427.27%, root fresh weight by 159.04%, shoot fresh weight by 115.22%, root dry weight by 23.08%, and shoot dry weight by 55.56% compared to without seed priming. Overall, this study provides practical recommendations: boron fertilisation can improve fruit quality, while TSP priming can improve seedling growth of GM-2 tomato.

Keywords: Boron fertilisation, fruit quality, GM-2 tomato, seed quality, seedling growth, trisodium phosphate priming

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INTRODUCTION

Tomatoes (*Solanum lycopersicum* L.) are a horticultural commodity that contributes significantly to global production. They are widely cultivated in regions with tropical and subtropical climates (Costa & Heuvelink, 2018; FAO, 2023). Tomato production and quality can be increased by using superior cultivars and improving cultivation management (García-Caparrós, 2024). GM-2 is one of the superior tomato accessions originating from Indonesia. The advantage of GM-2 is that they carry dominant genes conferring nematode resistance (Murti et al., 2012). A challenge that has emerged in recent years is that GM-2 accessions have shown a trend of declining seed and fruit quality, specifically indicated by low germination and vigour. This has implications for increasing tomato productivity. Yilmaz et al. (2017) and Zhou et al. (2019) reported that declines in seed and fruit quality are positively correlated with tomato yield.

Seed quality, pre-harvest, and post-harvest are key factors in successful tomato cultivation (Finch-Savage & Bassel, 2016; Kamran et al., 2020). Nutrient availability plays a crucial role in supporting reproductive development and seed formation (Ahmed et al., 2024; Marschner, 2012; Yousaf et al., 2016). Boron also plays a role in flowering initiation, supporting pollen germination, and maintaining the continued development of flowers and fruits (Thakur et al., 2023). Boron deficiency results in stunted root development, leaf shedding, and reduced plant yield (Aftab et al., 2022; Shireen et al., 2018). Other studies have reported that boron deficiency in several horticultural commodities (including tomatoes) can cause leaf chlorosis, stunted plants, low fruit set, and rough fruit surfaces (Chakraborty & Bose, 2023; Mal et al., 2025; Xu et al., 2021).

The study on boron fertilisation in tomatoes showed that it can improve vigour, increase vitamin C content, and extend fruit shelf life (Milagres et al., 2019; Rodriguez-Espinosa et al., 2020). Naz et al. (2012) found that boron fertilisation at 2 $kg.ha^{-1}$ significantly affected growth, flowering, and yield of tomato. Other functions of boron in several horticultural commodities include minimising flower drop due to environmental stress and increasing fruit set (Boldingh et al., 2016; De Silva et al., 2022). Seed quality can also be improved through seed priming. Seed priming is a method of seed hydration to improve germination and seedling emergence (Sharifi et al., 2016). The use of seed priming technology is due to its many advantages, including ease of use, affordability, time-saving, and proven results. Factors to consider for effective seed priming include optimal concentration and soaking duration (Afzal, 2023; Rifna et al., 2019; Saha et al., 2022). This relates to the induction of internal physiological and biochemical processes in seeds, thereby increasing germination, germination index, and yield (Ali et al., 2020; Devika et al., 2021).

The potential phosphorus-based priming agent is trisodium phosphate (TSP) (Baig et al., 2020). Córdoba-Sellés et al. (2007) reported that soaking tomato seeds in a 10% TSP solution for three hours increased germination. There are few studies examining

the effectiveness of TSP on horticultural commodities, particularly tomatoes. Furthermore, studies examining the combined effects of boron application in the pre-harvest phase and seed priming with TSP in the post-harvest phase on fruit quality, seed quality, and seedlings are still very limited, particularly in GM-2 tomato accessions. The hypothesis of this study is that boron application during the pre-harvest phase can increase yield components, yield, and seed quality. In contrast, seed priming with TSP can improve tomato seedling growth, especially in GM-2 accessions. The challenge of this study is whether these two approaches can interact positively to increase yield, seed quality, and the number of tomato seedlings.

This study is important because it provides information on an integrated approach combining pre-harvest and post-harvest technologies to improve tomato seed quality. This can provide practical recommendations for farmers, researchers, and seed producers on tomato seed and fruit production, especially for GM-2 accessions in Indonesia. The novelty of this study lies in integrating boron fertilisation and TSP seed priming to evaluate their combined effects on tomato fruit quality, seed quality, and seedling performance. Therefore, the objectives were to evaluate the effect of boron fertilisation on fruit quality and to assess the combined effect of boron fertilisation and TSP seed priming on seed quality and seedling growth in GM-2 tomatoes.

MATERIALS AND METHODS

Study Site

The study on boron fertilisation in a greenhouse in the Tejosari, Sub-district, Ngablak District, Magelang Regency, Central Java, Indonesia, was conducted from October to December 2023. This study site was situated at an elevation of about 1,350 meters above sea level, approximately 26 kilometres to the northeast of Magelang City, with geographic coordinates of 7°24'7" South Latitude and 110°24'5" East Longitude. The study on boron fertilisation and TSP priming was conducted in the greenhouse at the Faculty of Agriculture, Universitas Gadjah Mada, Special Province of Yogyakarta, Indonesia, from December 2023 to January 2024. The study site was approximately 140 metres above sea level and 5.3 kilometres northeast of Yogyakarta city, with the location coordinates 7°46'14" South Latitude and 110°22'39" East Longitude.

Experimental Design and Agronomic Practices

The study on boron fertilisation was carried out utilising a Completely Randomised Design (CRD) with three replications (Welham et al., 2015). Boron fertilisation consisted of 0 and 1.25 g.plant^{-1}. The selected tomato seedlings were planted in a greenhouse with beds measuring 4 m × 0.8 m. The seedlings were transplanted 25 days after sowing (DAS), with a spacing of 70 cm × 45 cm. Boron fertiliser was applied when the tomatoes reached

the generative phase, 8-11 weeks after transplanting (WAT). The boron dose given in this study was 1 kg per 200 L, equivalent to 1.25 g.plant^{-1}, dissolved in 250 mL of water. Harvesting was carried out four times at 14, 15, 16 and 17 WAT. Watering was carried out every two days until field capacity was reached (Panah Merah, 2024; Zharfa, 2024). Seeds were obtained from several harvest criteria (red colour) and from bunches two and three. Bapary et al. (2024) reported that tomatoes harvested at the red stage achieve the highest seed quality.

The study on boron fertilisation and TSP priming was conducted using a Randomised Complete Block Design (RCBD) factorial with three blocks as replications (Welham et al., 2015). Seeds produced from previous boron fertilisation studies (0 and 1.25 g.plant^{-1}) were soaked in TSP solution based on the treatments (0, 5, 10, and 15 g.L^{-1}) for one hour. The seeds were soaked for one hour and then washed with running water for 30 minutes (Thiruppathi et al., 2018; Zharfa, 2024). Seeding was carried out in trays filled with a planting mix of one part soil and six parts goat manure. We watered the trays every two days, and harvesting occurred after 21 days (Panah Merah, 2024; Zharfa, 2024).

Data Collection

Data collection for the study on boron fertilisation included macro- and microclimate, soil quality, and morphological variables. Macro and microclimate traits were total rainfall, air temperature, relative humidity, sunshine duration, and photosynthetically active radiation (PAR) (Albuja-Illescas et al., 2025; Alsamir et al., 2017; Šalagovič et al., 2024; Shamshiri et al., 2018). Soil quality traits were pH H_2O, soil organic carbon (SOC), cation exchange capacity (CEC), nutrient availability (phosphorus, potassium, and boron), and total boron (Soil Survey Staff, 2017; Sparks et al., 2020). Morphological traits were the number of fruits per plant, fruit length, fruit width, fruit fresh weight, fruit firmness, number of seeds per fruit, 1000-seed weight, and seed moisture content (Urban et al., 2017; Zharfa, 2024).

Data collection for the study on boron fertilisation and TSP priming included macro- and microclimate and morphological variables. Macro and microclimate traits were total rainfall, air temperature, relative humidity, sunshine duration, and PAR (Albuja-Illescas et al., 2025; Alsamir et al., 2017; Šalagovič et al., 2024; Shamshiri et al., 2018). Morphological traits were the germination, vigour index, leaf area, root length, root fresh weight, shoot fresh weight, root dry weight, shoot dry weight, and root-to-shoot ratio (Urban et al., 2017; Zharfa, 2024). Macro- and microclimate observations were conducted at the study site. Soil analysis was conducted at the Agricultural Instrument Standardisation Agency (BSIP) of the Ministry of Agriculture, Yogyakarta, Indonesia. Morphological observations were conducted at the study site and the Crop Production Management Laboratory, Department of Agronomy, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Statistical Analysis

Data from the study on boron fertilisation were analysed using the F-test at the 95% confidence level, followed by a Pooled T-test ($p < 0.05$). Data were visualised using box plots with error bars. Data from the study on boron fertilisation and TSP priming were tested for linear model assumptions, namely normality and homogeneity, using Q-Q plots and residual vs value graphs. Data that met the linear model assumptions were then analysed using a two-way ANOVA at a 95% confidence level, followed by an HSD-Tukey test ($p < 0.05$) (Welham et al., 2015).

Relationships between morphological traits were analysed using a correlation metric (Hadd & Rodgers, 2021). PCA-Biplot analysis produced a two-dimensional graph depicting the relationships between morphological traits (Gabriel, 1971). This was achieved by consistently allocating values to the components of the data matrix. The F-test, T-test, Q-Q plots, residual vs value graphs, ANOVA, and HSD-Tukey test were analysed using SAS® OnDemand for Academics software with proc mixed, while correlation metric, and PCA-Biplot using R software version 4.4.0 with the corrplot, FactoMineR, factoextra, GGally, ggplot2, pheatmap, and scatterplot3d packages (Kassambara, 2017; R Core Team, 2017; SAS Institute Inc., 2025; Sievert, 2020).

RESULTS AND DISCUSSION

Site Characteristics

The study on boron fertilisation reported total rainfall, average air temperature, relative humidity, sunshine duration, and PAR of 27.06 mm, 27 °C, 65.50%, 6.47 hours, and 11 $W.m^{-2}$, respectively. The study on boron fertilisation and TSP seed priming reported total rainfall, average air temperature, relative humidity, sunshine duration, and PAR of 617 mm, 25.95 °C, 85.00%, 8.65 hours, and 82.32 $W.m^{-2}$, respectively. In general, both study sites were suitable for tomato cultivation (Pashiardis et al., 2022; Shamshiri et al., 2018; Yang et al., 2022; Yousef et al., 2021; Zheng et al., 2023).

The soil type in the boron fertilisation study was classified as an Andisol. Andisol was formed from volcanic material rich in organic matter, dark in colour, and with a high capacity to retain water and nutrients. Andisol are known to have relatively high fertility, making it highly promising and widely used for the cultivation of horticultural commodities (Hati et al., 2021). Soil analysis results showed high SOC, a slightly acidic pH in H_2O, and a relatively low CEC. Available phosphorus and potassium were categorised as moderate to high, while total and available boron levels were very high. Total boron increased from 15.0 to 18.0 ppm (Table 1). This was influenced by volcanic activity in the surrounding area, which was surrounded by several active volcanoes (Rodriguez-Espinosa et al., 2020).

Table 1
Soil quality characteristics

Soil Quality	Unit	Initial	Final
pH H_2O	-	6.3	6.1
Soil Organic Carbon	%	6.10	6.20
Cation Exchange Capacity	$cmol^{(+)}.kg^{-1}$	7.52	8.28
Available of Phosphorus (Olsen)	$cmol^{(+)}.kg^{-1}$	18.15	12.91
Available of Potassium	$cmol^{(+)}.kg^{-1}$	5.01	4.42
Total Boron	ppm	15.0	18.0
Available of Boron	ppm	2.4	-

Effect of Boron Fertilisation on Fruit and Seed Quality

Boron fertilisation significantly affected tomato yield and quality, particularly fruit number and fresh weight, while fruit length and width were not significantly affected (Figure 1). Thakur et al. (2023) explained that boron fertilisation can improve fruit quality, including diameter and weight. Increased boron concentrations negatively affected morphological parameters, yield, and some fruit characteristics in tomatoes. Conversely, high boron concentrations were reported to increase fruit firmness, soluble solids content, and leaf B concentrations (Turhan, 2021). The results of this study also showed that the application of boron at 1.25 $g.plant^{-1}$, compared to the control, resulted in significantly higher fruit number (from 25.37 to 27.75 g, an increase of 9.38%) and fresh weight (from 184.12 g to 210.47 g, an increase of 14.34%). Hapuarachchi et al. (2022) stated that boron fertiliser, when applied correctly and at the correct dosage, can increase fruit weight, diameter, and quality.

Boron fertiliser at a dose of 1.25 $g.plant^{-1}$ resulted in fruit hardness of 35.80 N, an increase of 5.64% compared to the control, while seed moisture content was 12.93%, an increase of 1.02%. The number of seeds per fruit and 1000-seed weight did not differ significantly between boron treatments (Figure 2). This is consistent with the explanation by Harker et al. (2015), who stated that genetic and environmental factors control seed development. In this case, the number of seeds and 1000-seed weight are more influenced by genetic factors.

Boron fertilisation during the generative phase of tomato cultivation revealed several important findings, including that not all morphological traits showed significant differences. The relatively limited responses of some parameters were due to the already high soil boron content. Under such conditions, boron addition may not substantially alter plant physiological responses. The high boron content in the soil is due to the presence of active volcanoes, namely Mount Merapi and Mount Merbabu, in the study area. Volcanic activity naturally releases boron into the environment (Rodriguez-Espinosa et al., 2020).

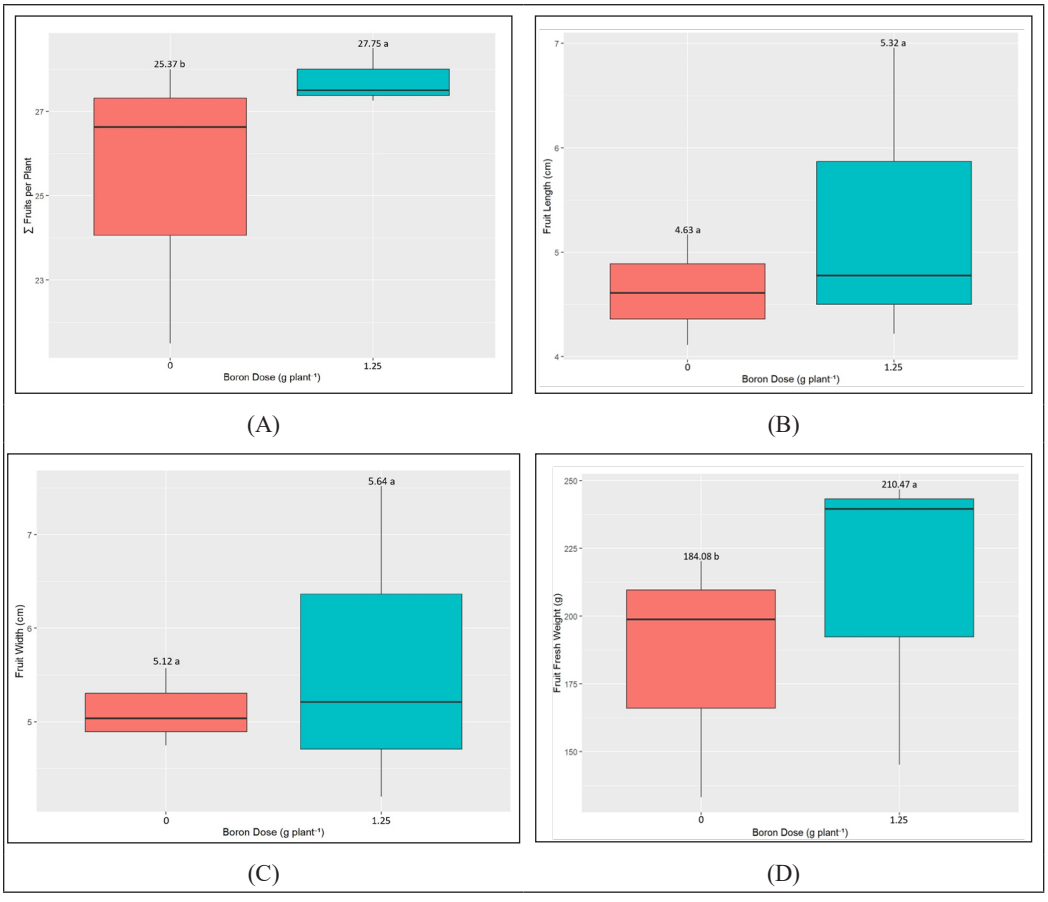


Figure 1. Boxplot of yield components and yield of GM-2 tomato affected by boron fertiliser. (A) Fruit number per plant; (B) Fruit length; (C) Fruit width; and (D) Fruit fresh weight

High soil boron levels at the study site did not cause toxicity in tomatoes. This is because tomato tolerance to boron ranges from 4 to 6 ppm (Flynn, 2015). Several variables observed in this study were not significantly different, possibly due to a calcium deficiency in tomatoes. Boron has antagonistic properties with calcium. Calcium deficiency can affect the quality of tomato fruit and seeds (Chockchaisawasdee et al., 2016; Correia et al., 2017; Galeriani et al., 2022; Matteo et al., 2022; Long & Peng, 2023).

Effect of Boron Fertilisation and TSP Priming on Seedling Traits

The vigour index is used to determine the rate of seed germination (Basu & Groot, 2023). There was no significant difference between boron fertilisation and TSP concentration in the vigour index. Boron fertilisation and TSP concentration did not show significant interactions in all observation variables. Boron fertilisation resulted in a germination rate of 89.16%, which was significantly lower than the 94.41% without boron fertilisation.

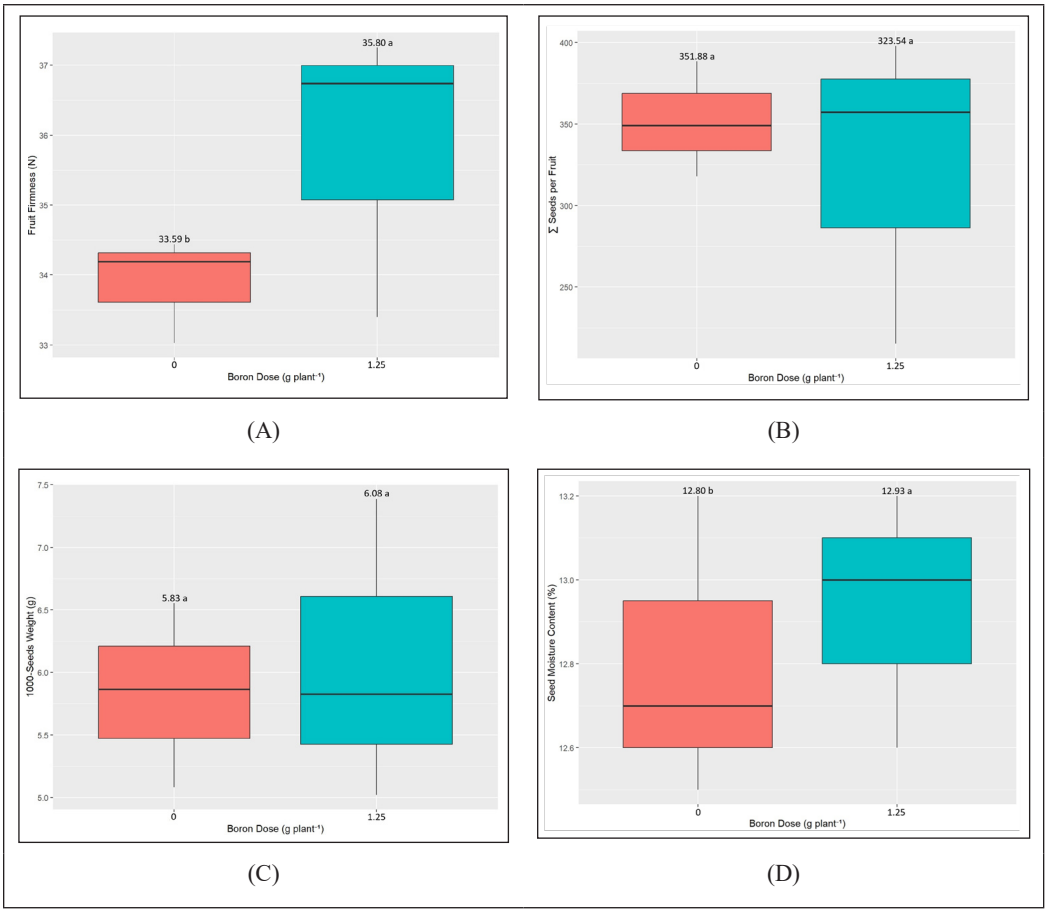


Figure 2. Boxplot of fruit and seed quality of GM-2 tomato affected by boron fertiliser. (A) Fruit firmness; (B) Seeds number per fruit; C) 1000-Seeds weight; and D) Seed moisture content

Boron application reduced germination by approximately 5.6%, whereas TSP concentration had no significant effect on germination (Table 2). Although the findings in this study indicate that boron fertiliser significantly affected germination, the resulting germination rates remained within the high range. Wijnker et al. (2025) stated that a high germination rate is an important indicator of seed quality (> 85% germination). Seed germination is influenced by species type and genetics, seed structure, and physiological properties (Forti et al., 2020; Sumbal et al., 2023; Wong et al., 2021).

Boron fertilisation of 1.25 g.plant^{-1} did not increase root length, leaf area, or root surface area (Table 2). Whereas TSP concentration significantly increased the root surface area. The TSP concentration of 15 g.L^{-1} resulted in a root surface area of 12.76 cm^2, a 427.27% increase compared to the control. Phosphorus availability enhances plant growth and development, particularly in apical meristematic tissue.

TSP can function as a growth regulator, and the phosphorus source provided can significantly alter root system architecture (Robles-Aguilar et al., 2020). Wen et al. (2017) reported that phosphorus application increased root length and root proportion in maize. Boron fertilisation had no significant effect on tomato biomass (fresh and dry weight). In contrast, TSP concentration showed a significant difference. The TSP concentration of 15 $g.L^{-1}$ tended to produce the highest biomass. Root fresh weight values ranged from 0.083 to 0.215 g, indicating a 159.04% increase compared to the untreated TSP concentration. Meanwhile, shoot fresh weight values ranged from 0.92 to 1.98 g, indicating a 115.22% increase compared to the untreated TSP treatment (Table 2).

Root dry weight and shoot dry weight values ranged from 0.026-0.032 g and 0.09-0.14 g, respectively. TSP application at the highest concentration increased root dry weight by 23.08% and shoot dry weight by 55.56%. Furthermore, the root-to-shoot ratio remained relatively stable across all treatments, ranging from 1.00 to 1.13 (Table 3). This indicates that the distribution of biomass between shoots and roots did not change significantly. Seed priming using phosphorus-based compounds can affect seedling development from the early stages of growth (Baig et al., 2020). This relates to the role of phosphorus in various important physiological processes. Razaq et al. (2017) stated that phosphorus has a function in cell division and elongation, regulating enzyme activity, and plant glucose metabolism.

Table 2
Germination, vigour index, leaf area, root length, and root surface area of GM-2 tomato were affected by boron fertilisation and TSP concentration in 21 days after seedling

Treatment	Germination (%)	Vigour Index	Leaf Area (cm^2)	Root Length (cm)	Root Surface Area (cm^2)
Boron Dose:					
0 $g.plant^{-1}$	94.41 ± 1.21^{a}	29.33 ± 0.65^{a}	29.7 ± 3.37^{a}	8.73 ± 0.82^{a}	7.67 ± 2.19^{a}
1.25 $g.plant^{-1}$	89.16 ± 1.90^{b}	26.93 ± 0.96^{a}	27.2 ± 4.14^{a}	10.12 ± 0.55^{a}	4.11 ± 1.30^{a}
TSP Concentration:					
0 $g.L^{-1}$	92.16 ± 2.79^{p}	28.37 ± 1.03^{p}	23.7 ± 5.85^{p}	10.06 ± 0.71^{p}	2.42 ± 0.63^{q}
5 $g.L^{-1}$	92.50 ± 1.57^{p}	28.89 ± 0.72^{p}	30.5 ± 2.79^{p}	9.05 ± 0.88^{p}	3.79 ± 1.30^{q}
10 $g.L^{-1}$	89.00 ± 3.46^{p}	27.36 ± 1.46^{p}	20.8 ± 3.03^{p}	8.47 ± 1.24^{p}	4.59 ± 0.99^{q}
15 $g.L^{-1}$	93.50 ± 1.82^{p}	27.89 ± 1.75^{p}	38.8 ± 6.09^{p}	10.11 ± 1.22^{p}	12.76 ± 3.86$_{p}$
Boron × TSP	(-)	(-)	(-)	(-)	(-)
CV (%)	1.43	3.34	18.26	10.37	32.25

Note. Mean ± SE values with different letters in the column indicate significantly different based on the HSD-Tukey test ($p < 0.05$); The (-) indicates no interaction between boron fertilisation and TSP concentration

Table 3
Root fresh weight, shoot fresh weight, root dry weight, shoot dry weight, and root-shoot ratio of GM-2 tomato affected by boron fertilisation and TSP concentration in 21 days after seedling

Treatment	Root Fresh Weight (g)	Shoot Fresh Weight (g)	Root Dry Weight (g)	Shoot Dry Weight (g)	Root-shoot Ratio
Boron Dose:					
0 g.plant^{-1}	0.132 ± 0.03^{a}	1.28 ± 0.15^{a}	0.027 ± 0.004^{a}	0.10 ± 0.011^{a}	1.11 ± 0.01^{a}
1.25 g.plant^{-1}	0.114 ± 0.02^{a}	1.31 ± 0.18^{a}	0.029 ± 0.004^{a}	0.11 ± 0.013^{a}	1.12 ± 0.02^{a}
TSP Concentration:					
0 g.L^{-1}	0.083 ± 0.02^{q}	0.92 ± 0.22^{q}	0.026 ± 0.008^{q}	0.09 ± 0.019^{q}	1.13 ± 0.03^{p}
5 g.L^{-1}	0.095 ± 0.01^{q}	1.25 ± 0.10^{q}	0.028 ± 0.003^{q}	0.11 ± 0.005^{q}	1.12 ± 0.02^{p}
10 g.L^{-1}	0.100 ± 0.01^{q}	1.04 ± 0.15^{q}	0.026 ± 0.004^{q}	0.09 ± 0.012^{q}	1.13 ± 0.02^{p}
15 g.L^{-1}	0.215 ± 0.05^{p}	1.98 ± 0.17^{p}	0.032 ± 0.007^{p}	0.14 ± 0.019^{p}	1.00 ± 0.04^{p}
Boron × TSP	(-)	(-)	(-)	(-)	(-)
CV (%)	3.26	11.00	0.75	1.89	2.34

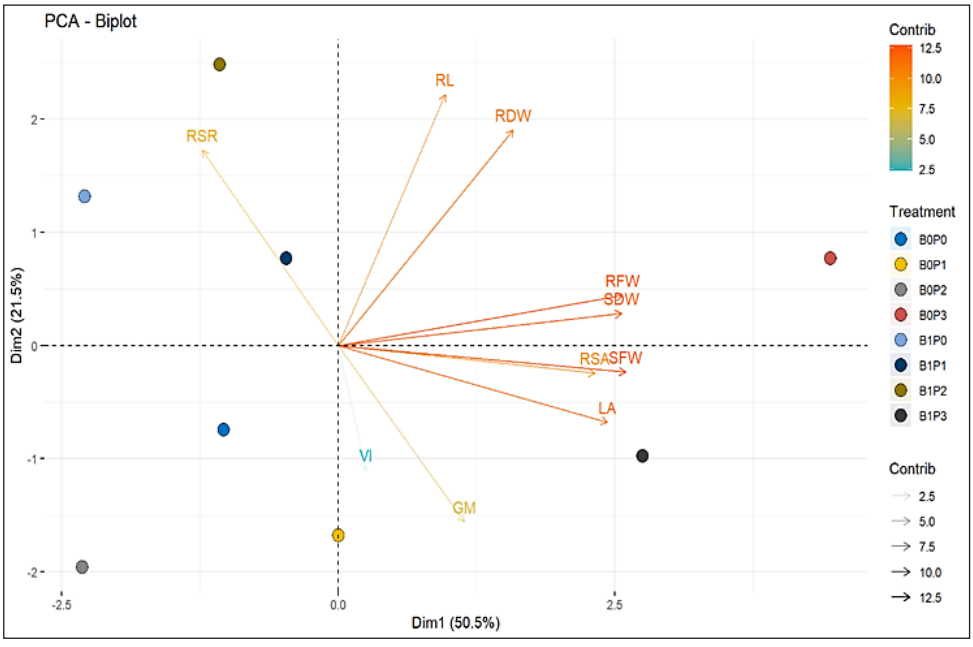
Note. Mean ± SE values with different letters in the column indicate significantly different based on the HSD-Tukey test ($p < 0.05$); The (-) indicates no interaction between boron fertilisation and TSP concentration

Relationship between Morphological Traits

Correlation analysis was used in this study to examine relationships among variables. Leaf area was highly correlated with shoot fresh weight (r = 0.865**) and shoot dry weight (r = 0.920**) (Figure 3A). Root and leaf structure play an important role in increasing plant biomass accumulation (Taraldsen, 2023). Improvement in root shape is positively correlated with increased fresh weight and dry weight of tomatoes (Ali et al., 2020). PCA-Biplot analysis is an effective approach for simplifying multivariate data without losing important information (Vafakhah & Janizadeh, 2021). In this study, the first two principal components explained most of the data variation, with PC1 contributing 50.50% and PC2 contributing 21.50% (Figure 3B). If examined further, the PCA-Biplot showed a fairly clear pattern of treatment groupings based on morphological characters, with four quadrants.

Quadrant I included B1P1, B1P0, and B1P2. This quadrant had similarities in root-shoot ratio (RSR). Quadrant II includes the B0P3. This quadrant was more closely linked to root growth and biomass. These included root length (RL), root fresh weight (RFW), root dry weight (RDW), and shoot dry weight (SDW). Quadrant III included B1P3. This showed a stronger pathway related to early growth performance. This included root surface area (RSA), shoot fresh weight (SFW), leaf area (LA), germination percentage (GM), and vigour index (VI). Quadrant IV, comprising B0P1, B0P0, and B0P2, showed no specific trends in morphological variables (Figure 3B).

(A)



(B)

Figure 3. Relationship between morphological traits: (A) Correlation matrix and (B) PCA-Biplot. B0 and B1 indicate without boron fertilisation (0 g.plant^{-1}) and boron fertilisation (1.25 g.plant^{-1}). P0, P1, P2, and P3 indicate Trisodium Phosphate (TSP) at the concentration levels of 0, 5, 10, and 15 g.L^{-1}

Wider leaves can improve the plant's ability to trap light. This contributes to increased tomato biomass accumulation. A good root system supports water and nutrient absorption, thus increasing the availability of materials for photosynthesis (Cochavi et al., 2020). The findings of this study indicate that boron fertilisation can improve traits related to fruit quality (number, fresh weight, and firmness). While, the TSP seed priming treatment had a greater impact on tomato seedling growth. Interestingly, there was no significant interaction between the two boron fertilisation treatments and TSP concentration. This study is important because it provides information on an integrated approach combining pre-harvest and post-harvest technologies to improve tomato seed quality. Overall, boron fertilisation and TSP seed priming can be applied to increase tomato production, especially for GM-2 accessions in Indonesia.

CONCLUSION

Boron fertiliser of 1.25 g.plant^{-1} significantly increased the number of fruits, fresh weight of fruits, fruit firmness, and seed moisture content by 9.38%, 14.34%, 5.64%, and 1.02%, respectively, compared to no boron fertilisation. There was no interaction between boron fertilisation and TSP seed priming treatment. TSP concentration of 15 g.L^{-1} increased root surface area, root fresh weight, shoot fresh weight, and shoot dry weight by 427.27%, 159.04%, 115.22%, 23.08%, and 55.56%, respectively, compared to those without TSP seed priming. Overall, boron fertilisation and TSP seed priming can be applied to increase tomato production. This study had limitations, namely, that it was conducted in soil with relatively high boron content. In addition, this study still focused on a single tomato accession, so conclusions about effects across various cultivars cannot be drawn.

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Optimising Glucomannan Production in *Amorphophallus oncophyllus* by Varying Tuber Size and Fertiliser Source

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ABSTRACT

Amorphophallus oncophyllus is widely cultivated in Indonesia to obtain starch and glucomannan content. The low productivity of the *A. oncophyllus* plant, which is only 6-12 tons ha^{-1} of fresh tubers, may be attributed to cultivation techniques that are still not intensive and remain dependent on natural harvest. The use of fertiliser type and tuber size is very important to increase production, as well as being efficient with the planting material used. The aim of this research is to evaluate the effect of inorganic fertiliser types (urea fertiliser, ammonium sulfate fertiliser and NPK fertiliser) and tuber size (whole tubers weighing 100 g, large tuber weighing 200 g (cut into 2 pieces), tuber 300 g (cut into 3 pieces) and tuber 400 g (cut into 4 pieces) on the growth and glucomannan content of *A. oncophyllus*. The results showed that statistical analysis showed no interaction between fertiliser type and tuber size treatments. Fertiliser type has an effect on stem diameter at 8 weeks after planting (WAP). Seed tuber portions influenced only the parameters of tuber number, tuber weight, and tuber diameter at the end of the observation period. The whole tuber treatment product relatively larger stem diameter at 11 weeks after planting (WAP), tuber diameter, and tuber weight compared with the half-tuber portion treatment. Our findings indicate that the whole tuber (100 g) has a relatively significant influence on tuber growth and glucomannan yield, providing important insights for commercial cultivation strategies.

Keywords: Glucomannan, inorganic fertiliser, tuber seeds

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INTRODUCTION

Amorphophallus oncophyllus is widely cultivated in Indonesia to obtain starch and glucomannan content (Nurlela et al., 2019). It has a polysaccharide substance consisting

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of randomly arranged α-mannose and α-glucose β-1,4. Glucomannan has broader benefits than cellulose and galactomanan. *A. oncophyllus* cultivation can be used as an alternative food and household food supply. People in mountainous areas usually plant *A. oncophyllus* under the canopy of trees or grow it wild without fertiliser input, but it is still useful for the economy.

There are fundamental things that need to be added as an effort to increase *A. oncophyllus* productivity. The average *A. oncophyllus* productivity is 6-12 tons ha^{-1} of wet tubers. Potential production can reach more than 40 tons ha^{-1} (Rahmawati 2023). The low productivity of *A. oncophyllus* plants is partly due to cultivation techniques that are still not intensive and still depend on natural harvests. *A. oncophyllus* cultivation techniques still need to be researched a lot, so agronomic improvements and planting materials still need to be reviewed, due to current conditions, the use of planting materials in farming is still inefficient.

The addition of inorganic NPK fertiliser is one way to improve agronomy in *A. oncophyllus* plants. Fertiliser dose of 50 kg N ha^{-1} and 100 kg ha^{-1} K2O to increase tuber weight 1 week after planting (Dewanti 2023). Potassium is the most important nutrient in all root crops for increasing yield and tuber quality (Torabian et al., 2025) and starch content (Berisha et al., 2014). Farmers in Amorphophallus cultivation prefer larger tubers as planting material so as to produce large tuber tillers. Plant growth is influenced by the size of the tubers, because the tuber contains food reserves that are useful for growth. Tuber planting material that is large in size can produce tuber weight and high glucomannan content.

Therefore, planting materials are used in large pieces of tubers. Planting material with the size of the whole tuber and the upper half with the main shoot produces larger tubers and plant height (Dewanti et al., 2023). However, research on different types of inorganic fertilisers and planting material sizes in Amorphophallus is still limited. The use of fertiliser type and tuber size is very important to increase production, as well as being efficient with the planting material used. The purpose of this research is to evaluate the effect of inorganic fertiliser type and tuber size on the growth and glucomannan content of *A. oncophyllus* plants.

MATERIALS AND METHODS

Experimental Site and Design

The experiment was conducted at the experimental Farm of the Faculty of Agriculture, VeteranNational Development University, East Java, Indonesia (at an altitude of 300 above sea level). Table 1 presents the set of experimental conditions used for the research. The study used a randomised complete block design (RCBD) with 2 factors (factor 1: fertiliser type and factor 2: seed tuber size) and 3 replications.

Table 1
Environmental conditions during the experiment

Variable	Environmental Conditions of Assessment
Average rainfall	255 mm
Minimum temperature	22^0 C
Maximum temperature	33^0 C
Average temperature	26^0 C
Average humidity	83%
Soil type	Lithosol
Altitude	300 asl

Note. Data are presented from the measurement results

The first factor used the types of inorganic fertiliser consisting of 3 levels: Urea fertiliser (Nitrogen 46%) 2 g/plant, Ammonium sulfate fertiliser (Nitrogen 21%, Sulfur 24%) 2 g/plant, and NPK fertiliser (Nitrogen 15%, Phosphorus 15%, Potassium 15%) 2 g/plant. Meanwhile, the second factor used tuber size of *A. oncophyllus*, namely whole tubers (100 g), large tubers weighing 200 g (cut vertically into 2 parts / 1/2 section), large tubers weighing 300 g (cut vertically into 3 parts / 1/3 section), and large tubers weighing 400 g (cut vertically into 4 parts / 1/4 section). These larger tubers are divided so that each piece weighs approximately 100 g. The data were obtained from 5 plant samples in each replication.

Planting Material and Preparation

A. oncophyllus tuber seeds were sourced from the area of Saradan Village, Madiun Regency, East Java (7°32′20″S 111°46′36″E). The tuber planting material is 1 year old (12 months). *A. oncophyllus* tuber seed used in the study consisted of whole tuber (100 g), large tubers weighing 200 g (cut vertically into 2 parts /1/2 section), large tubers weighing 300 g (cut vertically into 3 parts /1/3 section), and large tubers weighing 400 g (cut vertically into 4 parts /1/4 section). These larger tubers are divided so that each piece weighs approximately 100 g. Before planting, the part of the tuber that is split is smeared with rubbing ash from wood-burning waste to protect the exposed parts from rot caused by soil microorganisms. The way to plant tubers is to position the shoot at the top for both whole tubers and split tubers.

A. oncophyllus tubers were planted in a mixture of soil and leaf litter compost (1:1; v/v) with a polybag size of 35x35 cm filled to a maximum of ⅘ part. The planting distance between polybags was 50x50 cm with a planting depth of 15 cm. *A. oncophyllus* tubers that have been used have gone through a sorting process based on the weight of the treatment. The application of inorganic fertiliser treatments was carried out when *A. oncophyllus* had grown (at 4 and 10 weeks after planting). Fertilisation was done by placing the fertiliser in the soil around the *A. oncophyllus* stem.

Growth and Yield Observation

Observations of *A. oncophyllus* plants were carried out from 1 to 22 weeks after planting (WAP). Stem diameter variables were observed at the bottom of the plant, 5-7 cm from the soil surface (Figure 1). The number of bulbils was counted per plant manually when the bulbils had appeared. Tubers were harvested, and the weight of fresh tubers was continued to be measured at the age of 22 weeks as data on tuber growth and development. The weight of tuber flour is generated from weighing 0.5-1 g of the sample in a 250 mL glass cup. Then 50 mL of 96% alcohol was added and stirred for 1 hour.

Figure 1. Measurement of the stem diameter of *Amorphophallus oncophyllus* plants

Glucomannan Content Analysis

The glucomannan content is obtained from *A. oncophyllus* tubers, washed and cleaned of the soil attached, the skin is removed, and then shredded. Mix the grated tubers into 200 mL of 0.3% aluminum sulfate. Then stirred for 15 minutes in a water bath at 55 °C and diluted to three times the volume. Furthermore, the mixture was then centrifuged at 1500 x g for 30 minutes to produce a supernatant. The supernatant was mixed with 95% alcohol at a ratio of 1:1, and a clot was produced. Then, the clot was separated and dried in an oven at 45 °C for 24 hours. The dried clots were weighed. The ratio between the dried glucomannan lumps and the weight of the extracted *A. oncophyllus* tubers was calculated as the glucomannan yield (Wardhani et al., 2020).

Data Analysis

The data obtained were subjected to an ANOVA test. Variables that showed a significant effect due to treatment were tested using Least Significant Difference (LSD), $\alpha = 5\%$, using Microsoft Excel 2010 version.

RESULTS AND DISCUSSION

Figure 2 describes the growth stages of the vegetative phase of the *Amorphophallus oncophyllus* plant. It shows the 5 stages of vegetative development of *A. oncophyllus* tuber plants to be studied. The first stage, the seedling tubers after planting began to appear, with shoot and root growth during 3 weeks after planting (WAP) (Figure 2A).

The condition of the tuber seedlings is still the same at the beginning of planting, then shoot and root elongation begin to accelerate at the age of 5 weeks after planting (Stage 2) (Figure 2B). The tubers exhibited changes in shape and size, becoming smaller due to the translocation of photosynthetate for the development of stems, leaves, and root elongation at the age of 8 weeks after planting (Stage 3) (Figure 2C). The observations have been reinforced by Yadav and Singh (2024) that 70-170 days after planting is the period of stem elongation as sink organs. It's time for the source and sink transition. Stage 4 has begun the formation of tubers from assimilate producers (leaves) at the age of 12 weeks after planting (Figure 2D). Growth and enlargement of tubers have been seen at the age of 20 weeks (Stage 5) (Figure 2E).

There was a significant effect on the stem diameter of *A. oncophyllus* plants among each tuber division treatment at the age of 7-11 weeks after planting, although at the age of 12 weeks after planting, there was no difference (Table 2). Whole tubers produced a 32% higher stem diameter compared to split tubers at the age of 7-11 weeks after planting. Without paying attention to the whole tuber treatment, there was no significant difference in stem diameter between the split tuber 2, split tuber 3, and split tuber 4 treatments at the age of 7-9 weeks after planting. Intact tubers produced a petiole diameter of 31.6% greater in *A. oncophyllus* plants than the planting material of the halved tubers; moreover, the weight of each tuber is the same at 100 g.

Table 3 shows that NPK fertiliser produced the relatively highest number of *A. oncophyllus* bulbils at 1.67 compared to urea and ammonium sulfate fertilisers at the age of 12 weeks after planting. The bulbs parameter, with the addition of NPK fertiliser treatment, increased by 89.7% from the age of 7-12 weeks after planting. This is because NPK fertiliser contains phosphorus (15%), nitrogen (15%) and potassium (15%).

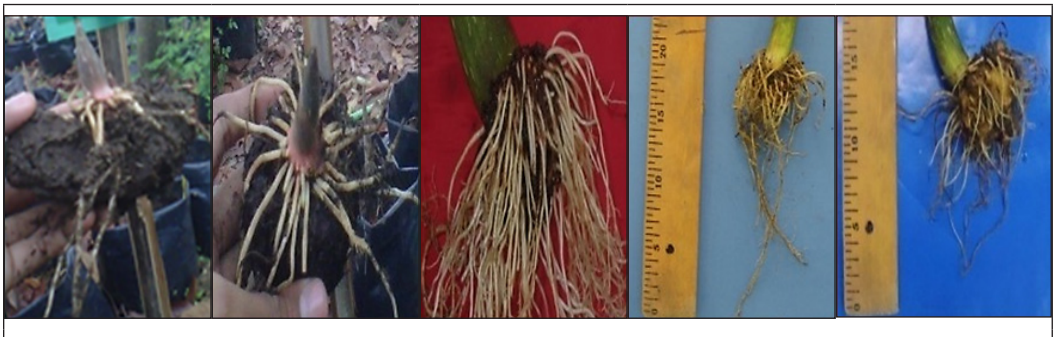

Figure 2. Developmental stages of the vegetative development cycle. (A) tuber seeds have started to appear shoots and roots at the age of 3 WAP; (B) elongation of shoots and roots at 5 WAP; (C) tuber shrinkage and formation of leaves, stems and roots at 8 WAP; (D) tuber formation at 12 WAP; (E) increase and growth of tubers aged 20 WAP

Table 2
Stem diameter of Amorphophallus oncophyllus plants with different types of fertiliser and portion of tuber seeds

Treatments	Stem Diameter (mm)					
	7 WAP	8 WAP	9 WAP	10 WAP	11 WAP	12 WAP
Inorganic Fertiliser						
Urea (P1)	2.67	3.57[b]	3.87	4.33	4.59	4.66
Ammonium Sulfate (P2)	1.83	2.62[a]	3.37	3.94	4.34	4.42
NPK (P3)	2.83	3.54[b]	3.64	3.88	4.17	4.04
LSD 5%	ns	0,88	ns	ns	ns	ns
Tuber Seeds						
Whole (U1)	3.64[b]	4.20[b]	4.51[b]	4.74[b]	5.00[b]	5.05
1/2 Section (U2)	2.31[ab]	3.05[a]	3.10[a]	3.49[a]	3.78[a]	3.90
1/3 Section (U3)	1.68[a]	2.68[a]	3.28[a]	3.94[a]	4.20[ab]	4.03
1/4 Section (U4)	2.15[a]	3.05[a]	3.62[a]	4.02[ab]	4.49[ab]	4.53
LSD 5%	1.41	1.07	0.85	0.79	0.84	ns

Note. Means followed by different letters in the same column indicated significant differences at LSD 5%. ns: not significant

Table 3
Number of bulbs of Amorphophallus oncophyllus plants with different types of fertiliser and portion of tuber seeds

Treatments	Number of Bulbs		
	10 WAP	11 WAP	12 WAP
Inorganic Fertiliser			
Urea (P1)	1.06	1.46	1.66
Ammonium Sulfate (P2)	0.80	1.16	1.58
NPK (P3)	0.88	1.46	1.67
LSD 5 %	ns	ns	ns
Tuber Seeds			
Whole (U1)	1.30[b]	1.97[b]	1.99
1/2 Section (U2)	0.76[a]	1.04[a]	1.55
1/3 Section (U3)	0.89[a]	1.14[a]	1.39
1/4 Section (U4)	0.71[a]	1.29[a]	1.61
LSD 5 %	0.39	0.56	ns

Note. Means followed by different letters in the same column indicated significant differences at LSD 5%. ns: not significant

Potassium content functions in the formation of bulbils, because potassium affects K + ions to bind water in plants and accelerate the rate of photosynthesis so as to stimulate the formation of bulbs (Johnson et al., 2022). Potassium plays a role in maintaining osmotic pressure and cell turgor so that water absorption in the transpiration process runs well (Supriyono et al., 2022). The element P also plays a role in carbohydrate and protein metabolism and the transfer of energy and carbohydrates from leaves to all parts of the plant, including bulbils (Wang et al., 2021). Phosphorus has a role in stimulating root growth, helping the fruit ripening process, helping plant respiration, and forming protein (Sardans & Peñuelas, 2021).

The formation of tubers through the process of cell development and multiplication will be followed by the enlargement of cells containing starch grains. The type of fertiliser did not have a significant effect on the variables of tuber diameter and tuber weight at the age of 16-22 weeks after planting (Table 4). The results of tuber weight in urea, ammonium sulfate, and NPK fertiliser treatments were 6.70 g, 6.94 g, and 5.58 g at the age of 22 weeks after planting. This is due to the fulfilment of the needs of essential elements of phosphorus and potassium, which play a role in the formation and enlargement of tubers in the process of starch synthesis. Potassium elements act as enzyme activators and affect the metabolic process of carbohydrate formation so that carbohydrates accumulate in tubers (Simelane et al., 2023).

Table 4
Tubers' diameter and weight of Amorphophallus oncophyllus plants with different types of fertiliser and portions of tuber seeds

Treatments	Corm Size	
	Diameter (cm)	Weight (g)
Inorganic Fertiliser		
Urea (P1)	1.71	6.70
Ammonium Sulfate (P2)	1.68	6.94
NPK (P3)	1.60	5.58
LSD 5 %	ns	ns
Tuber Seeds		
Whole (U1)	1.88 [b]	8.45[b]
1/2 Section (U2)	1.56 [a]	5.21[a]
1/3 Section (U3)	1.54 [a]	5.32 [ab]
1/4 Section (U4)	1.67 [ab]	6.65 [ab]
LSD 5 %	0.30	3.21

Note. Data are presented from the measurement results

The results of tuber diameter in the treatment of urea, ammonium sulfate, and NPK fertilisers were 1.71, 1.68 and 1.60 at the age of 22 weeks after planting. Tuber weight and diameter were significantly affected by the treatment of tuber division at the age of 19 to 22 weeks after planting (Table 4). Differences in weight and storage capacity of food reserves in *A. oncophyllus* tubers used as plant seeds (Soedarjo, 2021). The results of tuber weight in the whole tuber division treatment amounted to 8.45 g, quadruple division amounted to 6.65 g, and triple division amounted to 5.32 g. The rate of tuber weight gain in the whole tuber division treatment amounted to 5.32 g. The rate of tuber weight gain in the whole tuber division treatment was 26.7% at the age of 16-22 weeks after planting. The diameter of the whole tuber treatment was 1.88, which was relatively higher compared to the 1/4 section treatment, which was 1.67. The diameter of the whole tuber treatment was higher and significantly different from the two-split and three-split tubers. The increase in the diameter of the tubers from the whole tuber treatment was 11.2% higher than the rhombic treatment by 5.7% at the age of 16 to 22 weeks after planting. Whole-size tubers produced better diameter development and bulbil yield, but not significantly so at the end of the observation. Seed tuber size can affect food reserves used during early growth and embryo maturity at the point of growth. The size of the seed tuber will affect the provision of nutrients used in the formation of vegetative periods such as buds, roots, stems and subsequent growth (Triharyanto et al., 2022).

The presence of apical buds in intact tuber seedlings is important for the early emergence of buds to become leaves, stems and roots. When the apical bud of tuber seedlings is damaged, it can cause inhibition of growth and early development of stem diameter (Table 2). The presence of apical buds is very important for the initial growth of *A. oncophyllus* plants; if the buds have been damaged, it will inhibit the growth of the vegetative phase. The stem weight experienced greater enlargement in seedlings of whole tuber portions, and tuber pieces were also reported in *A. oncophyllus* plants (Dewanti et al., 2023). Moreover, research that has been conducted shows that the portion of tuber seedlings with damaged apical buds (due to splitting) produces a smaller stem diameter than intact tubers that have intact apical buds, resulting in a decrease in yield (Tables 2, 3, and 4). The results of this study are in line with Nurhalim et al., (2023) uncut or intact tubers independently produce more production than the sweet potato tuber cutting treatment. In addition, whole tubers with a weight of 100 g produce higher production compared to 100 g tuber pieces.

Utilisation of *A. oncophyllus* tubers into flour is one option to facilitate storage and further processing. Selected *A. oncophyllus* tubers are then sliced vertically as thick as (0.5-1 cm), followed by drying, which is called chip. *A. oncophyllus* flour contains a fairly high glucomannan content of 64.98%. The largest glucomannan content in *A. oncophyllus* flour ranges between 49-60% (Shi et al., 2019). Glucomannan is a polysaccharide consisting

of galactose, glucose, and mannose chain bonds. The main chain bonds are glucose and mannose, while the branch is galactose (Aanisah et al., 2022). Glucomannan is a water-soluble dietary fibre that is a strong hydrocolloid and low in calories. Glucomannan also has special physical properties, namely being able to expand in water up to 138-200% (Herawati et al., 2022).

There was no interaction between the type of fertiliser and the portion of tuber seeds on flour weight and glucomannan yield (Figure 3). Figure 3 shows that *A. oncophyllus* plants produce flour weight and glucomannan yield percentage that vary between 1.6-2.05 g and 9.65-41.80%. Two-split tuber seedlings with the addition of NPK fertiliser relatively increased 48.9% in flour weight compared to the addition of urea, thus affecting the amount of glucomannan yield of 41.80%.

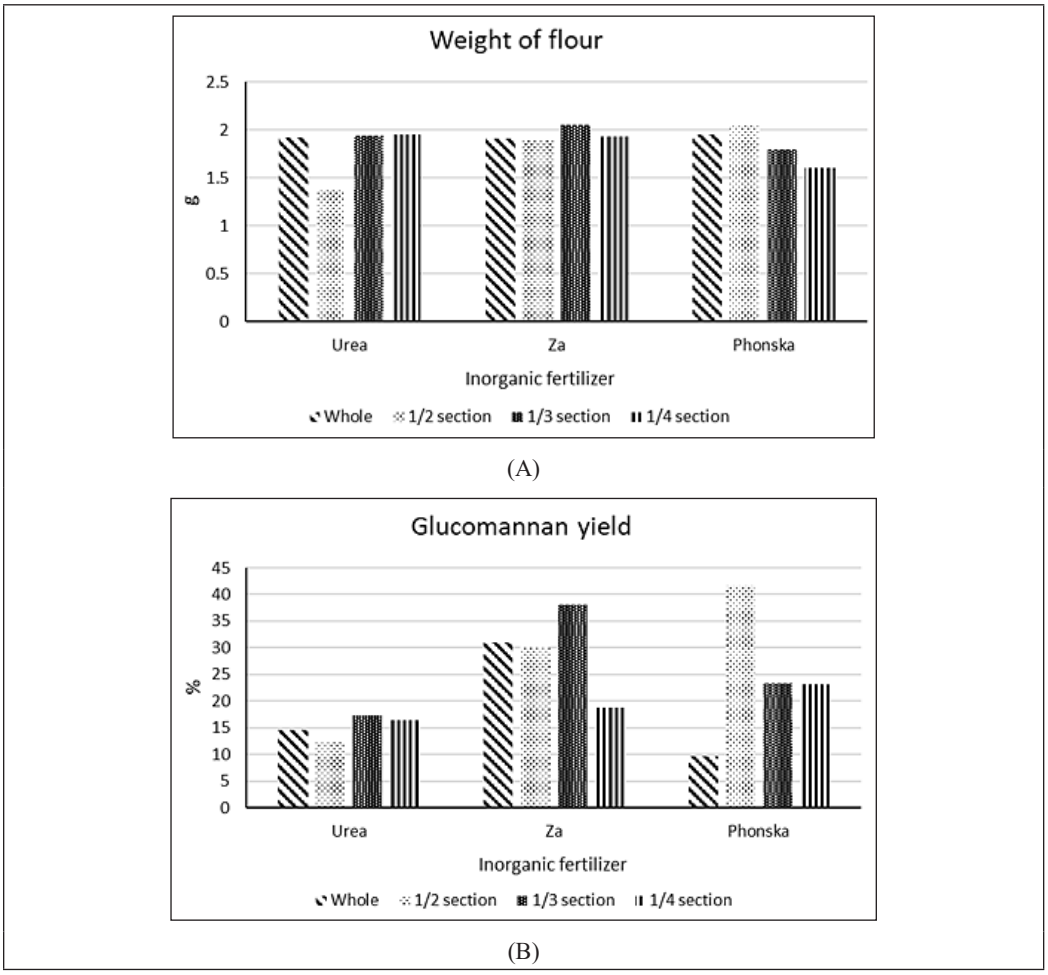

Figure 3. (A) Flour weight and (B) Glucomannan yield of *Amorphophallus oncophyllus* plants with different types of fertiliser and portion of tuber seeds

This shows that the two parameters are directly proportional, namely, the higher the weight of *A. oncophyllus* flour produced, will increase the amount of glucomannan yield content. Glucomannan has a strong relationship with yield (Setiavani & Suarti, 2023).This study has implications for the use of fertiliser types in order to agronomically improve the productivity of *A. oncophyllus*. Urea and NPK showed greater stem diameter growth than Ammonium Sulfate fertiliser at 8 weeks of age (Table 2). However, the application of fertiliser types showed similar results between treatments on the number of bulbils, diameter and weight of bulbs, flour weight, and glucomannan yield (Tables 2, 3, 4, and Figure 3). The application of urea fertilisers in this study relatively increased stem diameter, number of bulbs, and corm size, but the use of ammonium sulfate and NPK fertiliser relatively affected the weight of flour and glucomannan, which were higher compared to urea. It was also reinforced by Beja and Apelabi (2019) that the combination of urea, SP36, and NPK fertilisers gave the best results on the wet weight of sweet potato tubers compared to urea fertiliser alone.

Finally, the research also provides benefits in improving the cultivation of *A. oncophyllus* plants by using the portion of tuber seedlings to streamline planting materials. The use of seedling tuber portions can be recommended to produce the same flour weight and glucomannan yield as whole tuber planting material. However, the use of tuber seed portions needs to be considered again and done carefully so that the bud eyes are not damaged. In this study, the mechanisms observed due to the portion of tuber seeds, namely growth inhibition (Table 2) and reduction in crop yields (Table 4). In line with research by Hidayatullah et al., (2020) Plant morphology that has strong vigour will result in high production of taro tubers. However, the portion of tuber seeds has not been proven to produce heavy starch and glucomannan content (Figure 3). In contrast to Maretta et al., (2023) the diameter, length, and weight of taro tubers have a direct and indirect effect on glucomannan content. Further studies are needed to look at the *A. oncophyllus* plant tubers on the use of fertiliser types and portions of tuber seeds.

CONCLUSION

Portion of tuber seeds as planting material influenced only the parameters of bulb number, bulb weight and bulb diameter in the final observation. Meanwhile, the use of fertiliser types have affect to stem diameter 8 WAP. The size of the stem diameter at 11 WAP, tuber diameter and tuber weight in the whole treatment reached 89%, 21% and 62% higher than in the 1/2 section treatment. The type of fertiliser treatment showed that the stem diameter results at age 8 WAP were 36.2% better, as shown by the application of Urea fertiliser. Our results suggest that seed size significantly impacts tuber growth and glucomannan yield, providing crucial insights for commercial cultivation strategies.

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Drought Tolerance of Five Selected Rice (*Oryza sativa* L.) Genotypes based on Morphological Physio-biochemical and Phenotypic Polymorphism

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ABSTRACT

Droughts are a significant cause of reduced rice yields in South Asia, particularly in rainfed areas, where they affect over 23 million hectares of cultivated land. The prediction is that 15 million hectares of flooded-irrigated rice crops in Asia will become drought-prone by the end of 2025. This present study used a Malaysian commercial established variety, the MR219, which possesses high yield potential but is drought sensitive, as a recurrent parent and was screened with four selected genotypes (IURON 6, IURON 18, MR219-4, and DULAR) as donor parents of drought tolerance. The objectives of the present study are to screen and identify the most potentially tolerant rice genotypes and identify link foreground markers that respond to drought among parental plants. The sensitivity of rice genotypes to drought stress was observed, where DULAR recorded the highest score of leaf rolling, which was grouped in the most sensitive genotypes, followed by MR219 as susceptible genotypes; meanwhile, MR219-4 and IURON6 were intermediate ranking, while IURON 18 was less sensitive (moderately resistant) genotypes according to leaf rolling score which was negatively correlated with stomatal conductance. It was found that out of 21 linked/functional markers that were screened for polymorphism, five (RM201, RM28148, RM511, RM3392, and RM520) showed polymorphism between the two parents. The RM511 functional marker linked to IURON18 was tested and polymorphic between the two parents. The rice variety IURON 18 was identified as a valuable source for creating drought-resistant rice through marker-assisted backcrossing.

Keywords: Drought, drought-tolerant, rice (*Oryza sativa*), genotypes, morphology, physio-biochemical attributes, polymorphism

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INTRODUCTION

Rice is a major cereal crop, and Asia dominates the global rice production and consumption (Shah et al., 2024). Droughts are a significant cause of crop yield reduction in rainfed farming lands across South Asia, impacting an estimated 23 million hectares of cultivated fields. The prediction that 15 million hectares of flooded-irrigated rice crops in Asia will become drought-prone by the end of 2025 (Islam et al, 2023). The pre-flowering and grain filling stages are considered critical periods for rice, during which the plant is particularly susceptible to drought stress, which can significantly impact yield and quality. Rice is a water-intensive crop that requires ample water throughout its lifecycle (Yadav et al., 2023).

Adopting drought-tolerant rice varieties is crucial for stabilising food grain supply and improving agriculture in areas facing water scarcity (Hassan et al, 2023). The selection of crop varieties for drought-prone areas focuses on identifying genotypes that exhibit both high yields and drought tolerance. It is challenging to solely rely on yield as the sole measure of drought tolerance due to the complex nature of drought tolerance itself, including its heritability (Site Noorzuraini et al., 2021). Alternatively, some morphophysiological parameters like stomatal conductance, leaf water potential, tiller number, and leaf rolling score. These indices, and others, help breeders assess the performance of genotypes under both stress and optimal conditions, allowing them to select lines that maintain yield even in drought-prone environments (Fen et al., 2015). Further, screening with drought-linked SSR (Simple Sequence Repeats) markers was selected for parental survey for the identification of polymorphic foreground. SSR (Simple Sequence Repeats) markers are DNA sequences that are easily identified in a genome, making them relatively straightforward to use in genetic studies (Das et al., 2024; Dixit et al., 2015; Vikram et al., 2016).

Molecular markers have been regarded as an important tool in genetic variability studies and supersede morphological markers in the sense that their analyses are not influenced by the environment (Akos et al., 2019b). Various DNA or molecular markers are available for genetic studies in rice; however, microsatellite or simplesequence repeat (SSR) markers have been employed widely because they exhibit highallelic variability and can be easily magnified by PCR. In addition, microsatellite markers are numerous, broadly spread throughout the rice genome, species-specific, codominant, and naturally polymorphic in contrast with other DNA markers (Chukwu et al., 2020; Miah et al., 2013).

These studies used a long-established elite rice variety in Malaysia, MR219, which was once commonly widely cultivated by the local rice farming community due to its high yield potential but possessed drought sensitivity as a recurrent parent. The rice variety was screened together with four other genotypes (IURON 6, 18, MR219-4 and DULAR) to act as donor parents for drought tolerance. Therefore, the present studies were designed with the following objectives:

1. To screen and identify potentially drought-tolerant rice genotypes based on morpho-physiological responses under drought stress; and

2. To identify polymorphic SSR markers associated with drought tolerance for potential donor selection.

MATERIALS AND METHODS

Experimental Site

The experiment was conducted in the greenhouse condition with average temperature ranges from 30-to 35 °C and 22 °C to 24 °C during day and night, respectively. The average relative humidity ranges from 60% to 92%. The location of the experiment was in Ladang 16 (Latitude 2°59'31" N, longitude 101°43'59" E) at the Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. The experiment was conducted under greenhouse conditions to ensure controlled environmental variables and uniform drought stress imposition.

Planting Materials

The selected rice genotypes in this study consisted of an elite variety (MR219), two lines that were collected from the International Upland Rice Observational Nursery (IURON): IURON6 and IURON18, one local advanced mutant line (MR219-4) from Nuclear Agency Malaysia, and one drought-tolerant control variety (DULAR) obtained from Rice Germplasm Centre MARDI Malaysia. Table 1 shows the five rice genotypes and their origin that were examined in this study. All genotypes were planted in a small plastic pot with a diameter of 30 cm and a height of 30 cm. Three seedlings at 21 days old were transplanted in each pot filled with the third quarter of Tok Yong soil series, that having a clay loam texture containing 20% sand, 47% silt and 33% clay with a pH of 5.3 and 2.2% organic carbon. The soil was obtained from the rice-growing area of Kemubu Agricultural Development Authority (KADA), Kelantan, Malaysia. Soil texture was determined using the hydrometer method, while soil pH and organic carbon content were analysed following standard laboratory procedures.

Table 1
Designation of rice genotypes was used for screening

Code	Rice Genotype	Designation
V1	IURON 6	BP1976B-2-3-7-TB-1-1
V2	IURON 18	CT 15679-17-1-1-2-3-M
V3	MR219 mutant	MR219-4
V4	DULAR	IRGC32561
V5	MR219	MR219-Recurrent parent

Basal fertilisation application was applied following Kamarudin et al. (2018) at an equivalent rate of N:P:K (150:60:50 kg ha^{-1}) proportionally adjusted for pot culture conditions. Weed control can be achieved through various methods, including manual weeding for narrow-leaf weeds and herbicide application for broadleaf weeds. Integrated Pest Management (IPM) practices, including targeted chemical control, were employed when necessary.

Drought Stress Treatments

Drought stress was imposed by withholding irrigation at the active tillering stage (25 DAT) until plants reached a leaf rolling score of 4 based on the IRRI Standard Evaluation System (SES). Soil moisture tension ranged between -40 and -60 kPa, measured using tensiometers (Takemura DM 8, Japan) installed at a depth of 30 cm (Table 2). After the beginning of the re-irrigation, the plants were kept to normal irrigation until harvest at 45 DAT.

Table 2
Description of leaf rolling score

Scale	Leaf Rolling Score	Rate
0	Leaves healthy	Highly resistant
1	Leaves start to fold	Resistant
2	Leaves folding (deep V-shaped)	Moderately resistant
3	Leaves fully cupped (U-shaped)	Moderately susceptible
4	Leaves margins touching (O-shaped)	Susceptible
5	Leaves tightly rolled	Highly susceptible

*Source: International Rice Research Institute (2014)

Soil Moisture Measurement

Soil moisture was measured by using a soil moisture meter (HH2) every day (11 days) while drought stress treatment was started at 25 DAT until 36 DAT.

Morphophysiological Parameter

Leaf Area

Leaf area of rice plant was sampled once at harvest, 45 DAT. The samples were taken and separated into two parts: culm and leaf. Leaf area was measured using a LI-COR 3100 area metre. Before the leaf area was measured, the rice leaves were ensured not to roll to avoid invalid readings. The measurement unit was expressed in centimetre square (cm^2).

Leaf Elongation Rate

Leaf elongation rate (LER, mm day^{-1}) of individual third leaves was calculated as the slope of the linear regression line through the data points within the phase of linear increase in leaf length, where L_f (mm) is the final leaf length and LER (mm day^{-1}) is leaf elongation rate. Elongation of the third leaf was measured.

Tiller Number

The number of tillers was manually counted on 45 days after transplanting. Observation and calculation of tiller numbers were continuously conducted until the paddy reached 45 days after transplant (DAT).

Leaf Rolling Score

The leaf rolling score was recorded at mid-day, 11 days after stress inducement using the scales by referring to SES for rice of IRRI (2014) (Table 2) from 0 (flat) to 5 (tightly rolled).

Shoot Dry Weight

The rice plant was harvested 45 days after transplant and was partitioned into two parts, namely leaf and culm. Samples were labelled according to the treatment and replication. Then, the separated parts of culm and leaf were put inside a brown paper bag and were dried in the oven at 60 °C for three days. After 3 days, shoot dry weight of the rice sample was taken with an electronic balance. The measurement unit was in grammes (g).

Stomatal Conductance

The fully developed leaf located at the third from the top was selected for the measurement of stomatal conductance using a porometer. Each treatment was taken at 11 days after the stress treatment. The readings taken were recorded manually, which were expressed in $mmolm^{-2}s^{-1}$.

Abscisic Acid (ABA) Concentration

After approximately four weeks of growth, segments of leaf and root tissue (~200 mg dry weight each) were harvested, rinsed to remove soil residues, flash-frozen in liquid nitrogen, then freeze-dried and stored in a -80 °C freezer. The samples were subsequently sent to Lancaster University for the quantification of abscisic acid (ABA) concentrations in leaves (on day 9 and at harvest) and roots.

Following the protocol described by McAdam et al. (2016), the leaf and root samples were bead-milled (Qiagen, Hilden, Germany) using 3 mm beads at 25 Hz/s for 3 minutes.

In short, 200 mg of tissue was placed into a 2 ml microcentrifuge tube, to which 400 µl of ethyl acetate was added, and the mixture was thoroughly homogenised. The homogenate was then centrifuged at 13,000 X g for 10 minutes at 4 °C to isolate the supernatant.

The samples were analysed using high-performance liquid chromatography coupled with electrospray ionisation mass spectrometry (HPLC-ESI/MS). An Agilent 100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) was interfaced with an Applied Biosystems Q-TRAP 2000 mass spectrometer (Applied Biosystems, Foster City, CA, USA). Chromatographic separation was conducted at 35 °C using a 3 µm C18 column (100 X 2 mm). The mobile phase system consisted of two solvents: Solvent A and Solvent B, comprising acetonitrile and 0.1% formic acid, respectively. The gradient elution profile was programmed as follows: 5-60% Solvent B from 0 to 7.5 minutes, followed by 60-95% B from 7.5 to 10 minutes. The column was then flushed with 95% B for 3 minutes and subsequently re-equilibrated with 100% Solvent A for 10 minutes. The injection volume was 2 µl, and the flow rate was maintained at 4 ml/min.

Mass spectrometric detection was carried out using negative-mode electrospray ionisation (ESI). Instrument parameters were optimised using the Quantitative Optimisation tool in Analyst software by simultaneously infusing MS calibration standards via syringe pump and injecting standards into a 200 µl/min stream of a 1:1 mixture of Solvents A and B. The optimised settings were as follows: cone voltage, 40 V; capillary voltage, 3 kV; source temperature, 400 °C; desolvation gas flow rate, 900 L/h; and cone gas flow rate, 50 L/h.

Molecular Screening Method

DNA Extraction, PCR Conditions, and Electrophoresis Procedures

Healthy leaves were harvested from 14-day-old healthy seedlings. Immediately after collection, the leaves were carefully wrapped in aluminium foil, appropriately labelled, and then stored at -80 °C until DNA extraction. Total genomic DNA was extracted from leaf tissues using the method described by Doyle and Doyle (1990) with minor modification: Approximately 250 mg of leaf tissue was ground using a mortar and pestle as well as liquid nitrogen to a very fine powder. The powdered leaf samples were transferred into 2 ml micro-centrifuge tubes, and 1000 µl CTAB buffer and 3 µl of mercapethanol were incorporated into the tubes, respectively, and then the samples were shaken at 65 °C and at 500 rpm for 1 hour usingan Eppendorf thermo shaker. Subsequently, the samples were centrifuged at 500 rpm for 5 minutes with the aid of a refrigerated centrifuge machine. Thereafter, the total supernatants were moved into a new 1.5 ml tube and then 600 µl of chloroform: isomyl alcohol was incorporated and mixed by inverting. The samples were then centrifuged at 13,000 rpm for 5 mins in order to get a phase separation and then the upper phase was moved into a new 1.5 ml tube again. In addition, 600 µl of isopropanol

at 4 °C was incorporated and mixed carefully by inverting the tubes at least 50 times, and no vortex was used. The samples were then incubated for 30 minutes at -20 °C and centrifuged at 13,000 rpmfor 10 minutes. The supernatant was poured out, and the pellets were air-dried and rinsed by the addition of 600 µl of 75% ethanol 1 to 2 times until the white DNA fibre was seen. Finally, the DNA samples were subjected to 50 µl of TE buffer and 1 µl of RNase, and kept at -40 °C for future analysis. The quality of DNA was tested using NanoDrop2000 (Thermo Fisher Scientific Inc., USA), and the majority of the DNA samples were within the acceptable range of 1.8-2.0 for 260/280 and 2.0-2.22 for 260/230 (Jun-Yan et al., 2006).

The polymerase chain reaction (PCR) amplification was accomplished using thermal cycler (T100TM, Bio-Rad, UK) in 15 µl reaction and each individual component final concentrations were 7.5 µl Power Taq Master Mix (2x) (Bioteke Corporation, China), 4.5 µl nuclease-free water, 1 µl of 10 µM of forward and reverse primer and 1 µl and 1 µl of 70 ng genomic DNA, respectively. A touchdown PCR protocol was adopted as described by Shamsudin et al. (2016) for PCR amplification as follows: 94 °C for 5 minutes; followed by 10 cycles of 94 °C for 15 sec, 62 °C for 15 sec (decreasing 0.5 °C per cycle), then 72 °C for 15 seconds, followed by 30 cycles of 94 °C for 15 seconds, 52 °C for 15 seconds, then 72 °C for 15 seconds and, a final extension for 10 min at 72 °C followed by rapid cooling to 4 °C before analysis for foreground and background markers each. The entire process took a period of 1 hr 24 minutes.

The magnified PCR products were settled in a 3% w/v Metaphor Agarose gel with 10 µl gel dye (Gel view, 10 µl per 100 ml 1×TBE) (Bioteke Corporation, China) to determine amplicons in a 1×TBE buffered electrophoresis. The gel was carried out at 80 V for 1 hour. A 50 base pair ladder (GeneDireX, Inc., Taiwan, ROC) (reference ladder) appropriate for use in agarose gel electrophoresis as a standard molecular weight was adopted to detect the size of alleles of the magnified PCR products. Eventually, the bands were observed under ultraviolet light employing digital Molecular Imager® (GelDocTM XR, Bio-Rad Laboratories Inc., USA).

Genotyping of SSR Markers

SSR markers were selected based on previous reports of association with drought tolerance in rice. PCR amplification was performed following standard protocols and polymorphism information content (PIC) was calculated to evaluate marker effectiveness. The linked microsatellite markers, are employed to monitor the target locus which are polymorphic between the two parents, were used to determine the tightness of linkage (Hasan et al., 2015). Different alleles of the genotypes were scored using software (Image Lab 3.1) viz A and B. Allele scoring was performed using Image Lab 3.1 software with reference to a 50 bp molecular weight ladder to ensure accurate size determination, while the polymorphic

bands were only retained if they showed clear, reproducible differentiation between parental lines. Loci exhibiting inconsistent amplification or suspected null alleles across replicates were excluded from analysis to ensure scoring reliability.

Experimental Design and Statistical Analysis

The experiment was arranged in a completely randomised design (RCBD) with three replications, where each replication consisted of one pot containing three seedlings. In each replication, pots were arranged side by side with a spacing distance of 15 x 20 cm. Analysis of variance (ANOVA) was conducted for each parameter under investigation to assess the extent of variation attributable to genotypes, stress treatments, and their interaction. This analysis was performed using Statistical Analysis Software (SAS) version 9.4 to evaluate the degree of variability. The average, data span, relative standard deviation, and variability index were also determined for each parameter. Tukey's test was employed for mean comparisons, and correlations among traits were analysed using the CORR procedure in SAS.

RESULTS AND DISCUSSION

Soil Moisture

Figure 1 shows the changes in soil moisture over the period of soil drying. Drought stress conditions were observed once the soil moisture fell below 20% at day 11, after standing water had drained out. Meanwhile, during the treatment period, soil moisture in well-irrigated conditions was kept close to full volumetric saturation (100%) at all times. Plants were re-irrigated when check varieties reached the score of 4 for leaf rolling, and the soil moisture achieved below 20% showed that the genotypes experienced moderate drought stress during the experiment.

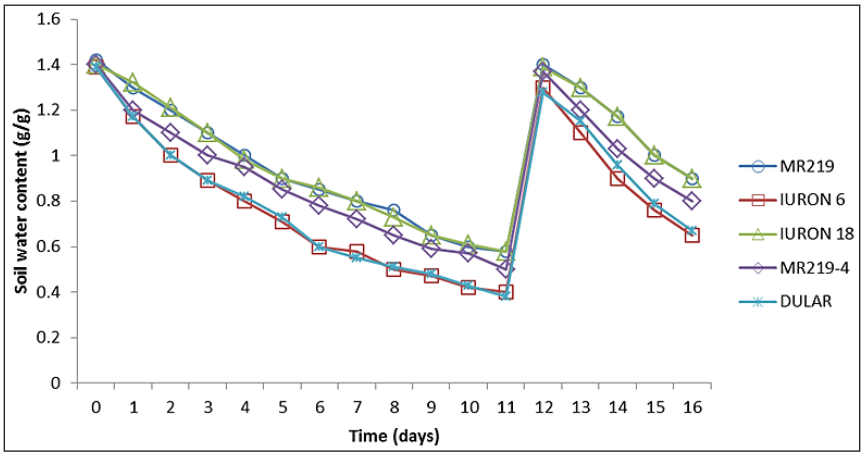

Figure 1. Development of soil water deficit and differences in soil moisture depletion rates among genotypes

Morphophysiological Traits

For the leaf rolling score, there was a statistically significant effect ($P<0.05$) of genotype, drought stress treatment, and their interaction. Similarly, data were recorded for leaf elongation rate, shoot dry weight, leaf area, stomatal conductance and leaf ABA concentration at day 9 drought stress was imposed. There was a significant difference for drought stress treatment ($P\leq0.05$) for leaf ABA concentration at harvest. Meanwhile, data were recorded with no significance of genotypes and their interaction of ABA concentration after re-irrigation. There were significantly different of genotype and drought stress treatment for root ABA concentration at day 9 drought stress was imposed; however, there was no interaction between genotype and treatment, similarly occurred for tiller number.

The reduction of tiller number, leaf elongation rate, leaf area, and shoot dry weight was detected under drought stress conditions compared to well-watered plants (Table 3 and 4). Leaf ABA concentration at day 9 after drought stress was imposed showed slightly higher values compared with well-watered plants. However, little increment was recorded in leaf ABA concentration at harvest, showing plants were not affected after re-irrigation. The value of stomatal conductance decreased until 90% under drought stress conditions, showing severe affected by drought stress (Table 3).

Percentage of Leaf ABA Accumulation Increment

Drought increases leaf ABA concentration by 183% at day 9 after drought stress was imposed (averaged across genotypes). IURON 6 and DULAR show the highest percentage of increment between well-watered and drought stress, meanwhile, IURON 18 showed the lowest increment percentage. On rewetting, leaf ABA concentrations were similar for both drought-stressed and well-watered plants. However, the percentage increment showed that IURON 6 and DULAR were significantly higher than IURON 18, MR219-4, and MR219. There is almost half as much (33%) increase in ABA concentration in the leaves of well-watered plants within a 4-day period between the drought cycle and harvest (Figure 2).

Leaf Rolling Score in Drought Stress

Leaf movement in rice was significantly affected by water stress. Leaf rolling reaction to drought stress was shown in Figure 3. According to the research, IURON 18 exhibited the least leaf rolling among the varieties, whereas DULAR recorded the highest leaf rolling rating on day 11 following the onset of drought stress. Leaf rolling scores were gradually increased at day 7 of drought stress imposition. Marked variations were observed among the genotypes and leaf rolling scores under drought stress conditions on day 8 after drought stress was applied. Plants were re-irrigated when a plant reached the score of 4 for leaf rolling, which was found at day 11.

Table 3
Means of leaf rolling score (for last day of drying cycle - day 9); leaf elongation rate; shoot dry weight; leaf area; stomatal conductance (day 9); leaf ABA concentration (day 9); leaf ABA concentration (at harvest); root ABA concentration; and tiller number under well-watered (WW) and drought stress (DS) conditions. Data are means ± SE for all irrigation treatments and all cultivars

Variable		IURON 6	IURON 18	MR219-4	DULAR	MR219
Leaf Rolling Score	WW	0.0±0.0	1.0±0.5	0.0±0.0	0.0±0.0	1.3±0.3*
	DS	3.5±0.6*	2.0±0.9	3.1±0.6*	5.0±0.0	3.4±0.4*
Leaf Elongation Rate (cm day^{-1})	WW	5.2±0.2	5.0±0.2	5.5±0.2	4.7±0.1	3.8±0.3*
	DS	3.0±0.4*	2.4±0.6*	2.8±0.4*	2.2±0.2*	2.6±0.2*
Shoot Dry Weight (g)	WW	5.9±0.3	3.8±0.2	5.5±0.3	6.1±0.5	3.8±0.2
	DS	3.9±0.4*	3.1±0.2*	3.7±0.4*	4.3±0.3	3.8±0.2
Leaf Area (cm^2)	WW	885.5±46.0	509.0±25.6*	763.5±46.0	1008.3±51.6	525.7±22.6*
	DS	512.9±10.5*	361.3±20.2*	451.9±10.5*	631.5±11.6*	415.5±26.2
Stomatal Conductance ($mmolm^{-2}s^{-1}$)	WW	216.6±17	114.9±24.8*	186.6±17	246.1±12.8	129.5±25.1*
	DS	54.6±17.4*	68.4±8.1*	61.6±17.4*	14.8±2.0*	60.6±12.2*
Leaf ABA Concentration - Day 9 ($ngg^{-1}dwt$)	WW	376.8±36.4.	332.1±38.7	439.0±33.5	251.2±24.5	439.0±32.6*
	DS	928.7±70.6	576.8±76.4*	1104.7±75.1	1028.7±150.6	921.0±97.8
Leaf ABA Concentration - at harvest ($ngg^{-1}dwt$)	WW	747.0±152.7	644.1±66.4	774.0±127.5	347.0±30.0	792.6±44.7
	DS	821.1±58.6	807.4±63.9*	802.1±86.5	562.1±53.9	844.3±34.6*
Root ABA Concentration - Day 9 ($ngg^{-1}dwt$)	WW	75.1±19.2	112.6±26.0	66.8±16.1*	117.0±16.7	55.1±12.8*
	DS	172.7±31.3*	155.1±30.0	131.8±30.0	278.0±32.1	128.7±13.3*
Tiller Number	WW	11±0.49	8.00±0.50*	11±0.49	19.00±1.17	10.00±0.3*
	DS	8.00±0.44	9.00±0.53	8.00±0.44	15.00±0.63*	7.00±0.74

Table 4
Drought tolerance ranking of genotypes

Scale	Rate	Leaf Rolling	Genotype
0	Highly Resistant	No symptoms of stress	
1	Resistant	No rolling	
2	Moderately Resistant	Partially rolled, unrolled in the evening	IURON18
3	Moderately Susceptible	Partially, unrolling in the late evening and early morning	IURON6, MR219-4
4	Susceptible	Complete, unrolling in the morning	MR219
5	Highly Susceptible	Like a tube; no unrolling in the morning	DULAR

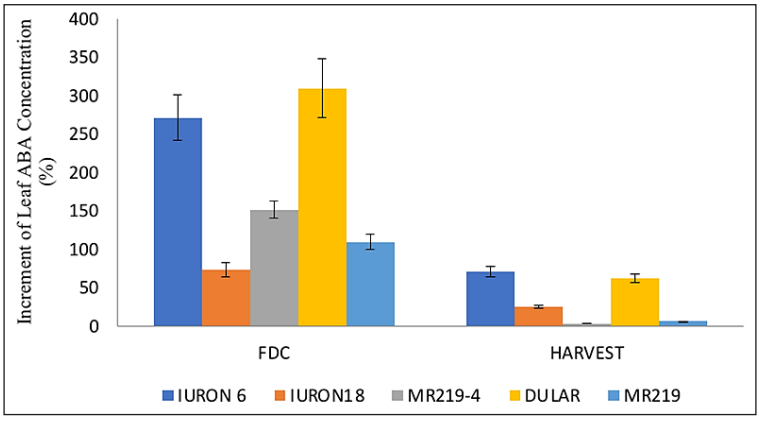

Figure 2. Percentage of leaf ABA accumulation increment between well-watered and drought stress at day 9, first drying cycle (FDC) and at harvest (after rewetting). Bars represent means ±SE of 3 replicates

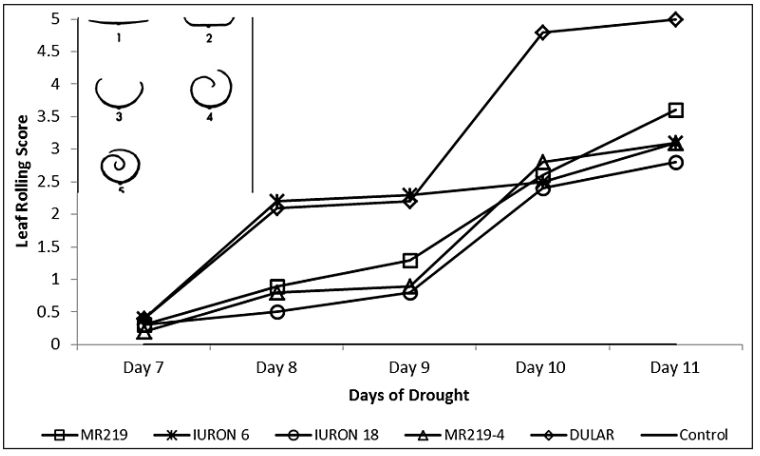

Figure 3. Leaf rolling measurements of all genotypes under drought conditions. Symbols represent means ±SE of leaf rolling scores

On the other hand, IURON 18 had the highest performance in drought tolerance ranking by moderately resistant, followed by IURON 6, and MR219-4 are moderately susceptible, and MR219 is susceptible rice to drought stress; meanwhile, DULAR is highly susceptible to drought stress. This indicated that IURON 18 had drought-tolerant properties which could be used as a donor for breeding purposes. A key indicator of drought in plants is leaf rolling, which is regarded as a crucial characteristic in rice breeding. Intense leaf rolling adversely affects plant growth, development, and grain production. However, moderate leaf rolling helps minimise water loss by decreasing the transpiration rate, thereby supporting plant survival under drought stress. Hence, regulating leaf rolling is a vital approach for enhancing rice yield during drought stress (Li et al., 2019). Because of its agricultural significance, leaf rolling has attracted considerable interest from plant researchers.

Relationship between Leaf Rolling and Stomatal Conductance

A negative linear relationship occurred between stomatal conductance and leaf rolling score, as shown in Figure 4. When the leaf rolling score increased, the stomatal conductance linearly decreased. Stomatal conductance decreased drastically with the increase in the degree of drought stress. The increase in leaf rolling score showed an increase in drought stress level. The equation for the negative linear relationship is shown below in Equation 1:

$$\text{Stomatal conductance} = -14.885x + 83.915 \text{ with } R^2 = 0.7067 \ (n = 15) \qquad [1]$$

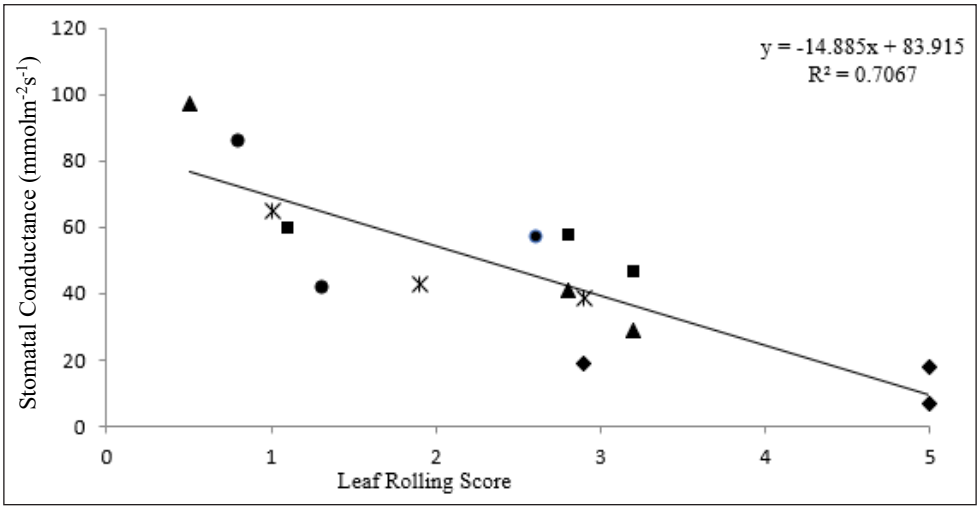

Figure 4. Relationship between leaf rolling and stomatal conductance (daily averages for three days. Star = IURON6; circle = IURON18; triangle = MR219-4; diamond = DULAR; square = MR219. Error bars omitted for clarity

Correlation between Morphological Variables in Response to Drought Stress

Leaf area showed a positive association with leaf elongation rate (r = 0.44) and tiller number (r = 0.86). At the same time, leaf elongation exhibited a positive relationship with leaf area (r = 0.44) and dry biomass (r = 0.36). However, leaf elongation rate is a poor predictor of important yield components (leaf area and dry mass). Meanwhile, leaf area is a better predictor of these yield components under drought stress. Tiller number was positively correlated with leaf area (r = 0.86) and dry biomass (r = 0.82). There was a positive correlation of dry biomass with leaf area (r = 0.94), leaf elongation rate (r = 0.36) and tiller number (r = 0.82) as shown in Table 5.

Table 5
Correlation table of morphological characteristics following drought exposure

	Correlations			
	Leaf Area (cm^2)	**LER ($cmday^{-1}$)**	**Tiller Number**	**Dry Biomass (g)**
Leaf area (cm^2)		0.44**	0.86**	0.94**
LER ($cmday^{-1}$)	0.44**		0.11	0.36**
Tiller number	0.86*	0.11		0.82**
Dry biomass (g)	0.94	0.36**	0.82**	

Note. * Correlation is significant at the 0.05 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)

Molecular Screening

In this study, five simple sequence repeat marker (SSR) were used to determine the polymorphism between MR219 as the recipient and other genotypes (IURON 6, IURON 18, MR219-4, DULAR) that will be chosen as the donor (Figure 5). Based on the gel images, the results showed IURON 18 distinguishing the polymorphism for five SSR foreground markers, namely RM201, RM28048, RM520, RM3392, and RM511. IURON18 will be selected as a donor parent because of carrying the character of interest. Foreground molecular markers showing polymorphism between the two parents, MR219 and IURON18, for the various target genes: RM511 ($qDTY_{12.1}$) is linked to increased harvest index, biomass yield, plant height and number of days to flowering, and RM520 ($qDTY_{3.1}$, $qDTY_{3.2}$) is associated with leaf dry weight, stem dry weight, total shoot dry weight, and leaf rolling score.

Another factor in selecting appropriate donors for developing drought-tolerant rice varieties was the identification of quantitative trait loci (QTL). According to Site Noorzuraini et al. (2021), pinpointing genomic regions associated with desired traits and performing indirect selection can be achieved without relying on complex phenotypic assessment. QTLs have been identified with significant and stable impacts on yield under drought conditions (Venuprasad et al., 2012) and have been found in various drought-tolerance related traits in rice (Akos et al., 2019a; Mohd Ikmal et al., 2019). Although drought resistance traits can be identified through QTL mapping, it remains necessary to evaluate and validate the effectiveness of these QTLs on grain yield under drought conditions in the field. For molecular screening, it was found that out of 21 linked/functional markers that were screened for polymorphism, five (RM201, RM28148, RM511, RM3392, and RM520) showed polymorphism between the two parents. The RM511 functional marker (Dixit et al. 2014) linked to IURON18 was tested and polymorphic between two parents. Although the use of functional/QTL-linked markers (RM511, RM520) may reduce null allele frequency due to conservation of primer-binding regions in drought-related genomic regions. This result corroborates the finding of Akos et al. (2019b), where their

improved varieties were developed and confirmed genotypically with drought tolerance QTLs ($qDTY_{12.1}$). Meanwhile, drought-tolerant alleles for QTL ($qDTY_{3.1}$ and $qDTY_{3.2}$) were confirmed using the functional marker RM520 (Singh et al. 2016) reported demonstrates a substantial positive contribution to grain yield under harsh drought stress in upland and lowland environments. $qDTY_{3.2}$ is one of the known Mega-QTLs (MQTL) and one of the large-effect QTLs was identified as the most precise and consistent across the environments and useful for Marker Assisted Selection (MAS), candidate gene identification and functional analysis (Kumar et al., 2014). This study reveals IURON 18 as the most promising genotype, showing superior performance in morphophysiological traits under drought environment in relation to parental polymorphism.

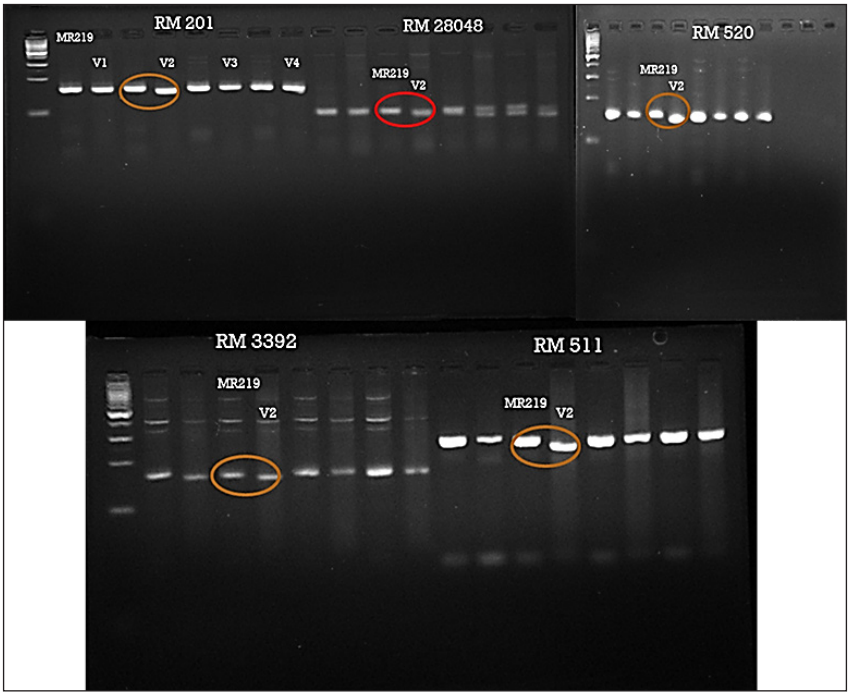

Figure 5. Screening of parental lines, IURON 6 (V1), IURON18 (V2), MR219-4 (V3), DULAR (V4), and recipient parent MR219, for polymorphism using some of the SSR markers linked with drought tolerance genes. Running on 3% metaphor agarose gel stained with midori green. M: 50bp Ladder

CONCLUSION

The study revealed substantial differences among the selected rice genotypes for the majority of the traits evaluated. In general, drought stress imposed significantly reduced leaf area, leaf elongation rate, shoot dry weight, and tiller number. In contrast, drought increases leaf ABA concentration by 183% at day 9 after drought stress was imposed

(averaged across genotypes). IURON 6 and DULAR show the highest percentage of increment between well-watered and drought stress, meanwhile, IURON 18 showed the lowest increment percentage. Leaf movement in rice plants was markedly influenced by drought stress. The present study showed that IURON 18 exhibited the least leaf rolling among the genotypes, whereas DULAR recorded the highest leaf rolling score at day 11 after drought stress was imposed. Moreover, IURON 18 exhibited the greatest performance on morphophysiological traits under drought environment in relation to parental polymorphism. Based on the results, we concluded that IURON18 was moderately resistant in drought tolerance ranking, followed by IURON6, MR219-4, and MR219, and DULAR were highly susceptible to drought stress.

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Review Article 937
Life Cycle and Feeding Behaviour of Rice Stem Borer Species in Southeast Asian Regions' Paddy Fields
Aisyah Pratiwi Suryanto Sugian, Patricia King Jie Hung, Muhamad Azmi Mohammed, Tunung Robin, Leong Sui Sien, Freddy Yeo Kuok San, Zakry Fitri Ab. Aziz, Noorasmah Saupi, Shiamala Devi Ramaiya, Ribka Alan, Adrian Daud, Anita Rosli, Philip Lepun, Zahora Ismail, Sivasangar Seenivasagam, and Suziana Hassan

Effect of Boron Fertilisation and Trisodium Phosphate (TSP) Priming on Fruit and Seed Quality of GM-2 Tomato (*Solanum lycopersicum* L.) 957
Amira Mufida Zharfa, Rudi Hari Murti, and Budiastuti Kurniasih

Optimising Glucomannan Production in *Amorphophallus oncophyllus* by Varying Tuber Size and Fertiliser Source 985
F. Deru Dewanti, Ramdan Hidayat, Careca Sepdihan Rahmat Hidayatullah, and Oentari Prilaningrum Sutanto

Drought Tolerance of Five Selected Rice (*Oryza sativa* L.) Genotypes based on Morphological Physio-biochemical and Phenotypic Polymorphism 997
Noor Asmaa Awang, Mohd Razi Ismail, and Zulkarami Berahim

Selangor Public Perceptions of Neutering and Preventive Health Measures for the Health of Cats and Dogs 775
Nur Athirah Nabila Jamaluddin, Michelle Fong Wai Cheng, Tengku Rinalfi Putra Tengku Azizan, Muhd Khaizer Omar, and Syamira Syazuana Zaini

Review Article 791
The Ecology and Conservation of Orchids in Ultramafic Habitats of Kinabalu Park, Sabah, Malaysia
Harniza Izzati Razali and Siti Fatimah Md.-Isa

Review Article 841
A Review on the Optimisation of Enzymatic Treatment in Tropical Fruit Juice: Impacts on Physicochemical and Functional Properties
Hasdarahayu Mohd Hassan, Mohd Azrie Awang, Ahmad Hazim Abdul Aziz, Asep Awaludin Prihanto, Abdul Aziz Jaziri, and Siti Faridah Mohd Amin

Abscisic Acid Exogenous Spraying to Improve Sorghum (*Sorghum bicolor* (L.) Moench) Genotypes for Tolerance to Abiotic Stress 863
Abdullah Amar Saleh, Yaseen Obaid Noori Ahmed Sharif, and Ali Hussein Raheem

Diversity and Ecological Insights of Fruit Bats in Durian Orchards of Sarawak, Malaysian Borneo 881
Sara Dalton Saging, Muhd Amsyari Morni, Wong Sin Yeng, Abd Rahman Mohd-Ridwan, Faisal Ali Anwarali Khan, and Jayasilan Mohd-Azlan

Phylogeography and Genetic Structure of the Giant Freshwater Prawn (Decapoda: Palaemonidae) in Peninsular Malaysia using Mitochondrial DNA Analysis 901
Dania Aziz, Afrina Irdina Roszizul Kahairi, Athirah Mohd Bakri, Nur Syuhada Mohd Fuad, Izzah 'Atirah Mohd Hir Tarmidi, Yuzine Esa, Md. Lifat Rahi, and Muhammad Dailami

Application of Multi-level Factorial Design in Optimisation of Ultrasound-assisted Extraction (UAE) Condition from *Erythrina variegata* L. Leaves on Antioxidant Activities 919
Nur Fazira Abdul Rahim, Norhayati Muhammad, Fazleen Izzany Abu Bakar, Mohd Fadzelly Abu Bakar, Siti Fatimah Sabran, Furzani Pa'ee, Balkis A. Talip, Ain Zamira Muhamad Zamri, Rozaliana Ab Karim, Nur Zainatul Nadra Zainol, Aliff Hisyam A Razak, and Rabiah Ahmad

Using Spectral Vegetation Indices to Study the Growth of *Opuntia ficus-indica* and Identify Suitable Production Sites in the Southwestern Region of Saudi Arabia 587
Abdulmalek A. Muharram, Elkamil Tola, Rashid S. Al-Obeed, Fahad Al-Qurainy, Khalid A. Al-Gaadi, Rangaswamy Madugundu, Ahmed M. Zeyada, Mohamed K. Edrris, Nabil Mohammed, and Abdulhakim A. Aldubai

Early Performance Results of Polyhalite on Newly Planted Oil Palms 607
Ng Fang Wen, Siti Hajar, and Lim Jing Jie

Valorisation of Kopyor Coconut Waste for Fatty Acid Production by *Yarrowia lipolytica* 627
Firda Dimawarnita, Muhammad Fayyadh Altamis Erwinsyah, Puspo Edi Giriwono, Yora Faramitha, Irma Kresnawaty, Urip Perwitasari, Titin Haryati, and Ario Betha Juanssilfero

Phenological Growth Stages of *Artocarpus odoratissimus* using the Extended BBCH Scale and its Applications in Determining Optimal Harvest Maturity 647
Isniti Richard, Halifah Afiza Ismail, Shiamala Devi Ramaiya, Gerevieve Bangi Sujang, Nuratika Tamimi Sheikh Mohamed, and Kundan Kishore

Enhancing the Physical Properties of Egg Custard-based Diets through Feed Supplements to Improve the Growth Performance of *Macrobrachium rosenbergii* Larvae 665
Riyad Taguemount, Jarunan Pratoomyot, Andrew P Shinn, and Rasina Rasid

Review Article 689
A Review of Key Factors Influencing Forest Fire and Wildfire Susceptibility: The Role of Climatic, Environmental, Topographic, and Anthropogenic Variables
Yee Jian Chew, Shih Yin Ooi, Ying Han Pang, and Zheng You Lim

Morphological Differences of Experimental Hybrids and Check Varieties of High-yielding Grain Maize 715
Siti Norfaroha Ariffin, Juju Nakasha Jaafar, Ghizan B. Saleh, Nazatul Shima Naharudin, and Samsuri A. Wahid

Unravelling the Diversity of *Azolla cristata* from the Progo and Opak River Basins based on Inter-simple Sequence Repeat Marker and Morphological Analysis 733
Nafiah Khoirunnisa, Adib Fakhruddin Yusuf, Aji Sukma Iqbal Najibulloh, Abdul Razaq Chasani, Rina Sri Kasiamdari, and Budi Setiadi Daryono

Review Article 761
Reproduction Technology for Sustainable Portunid Crab's Aquaculture: A Review
Ariffin Hidir, Mohd Amran Aaqillah-Amr, and Mhd Ikhwanuddin

Pertanika Journal of Tropical Agricultural Science
Vol. 49 (2) Apr. 2026

Content

Foreword i
Md Kamal Uddin

Morphological Variation of Liberica Coffee (*Coffea liberica* Hiern) in Kalipuro Sub-district, Banyuwangi Regency 449
Naila Izzatul Mukhoyyaroh, Rodiyati Azrianingsih, Estri Laras Arumingtyas, and Luchman Hakim

Analogue Rice from *Amorphophallus muelleri* and Potato with Enhanced Nutritional Properties: Lower Glycemic Index and Higher Fibre Content 473
Kisroh Dwiyono, Nor Elliza Tajidin, Maman Abdurachman Djauhari, Elisa Azura Azman, Nur Syahirah Abdul Rashid, Viktor Vekky Ronald Repi, and Noverita

The Influence of Zinc and Boron Application on Growth, Yield, Seed Quality, and Nutrient Content in Non-hybrid Corn 487
Agustiansyah, Paul Benyamin Timotiwu, Eko Pramono, Galuh M. Pramudyano, and David Chandra

A Second Life for Agro-industrial Residues: Edible Coating from Roselle Decoction Residues for Preserving Wax Apple Quality and Antioxidant Properties 497
Nur Amirah Yusoff, Fauziah Tufail Ahmad, Aidilla Mubarak, Razifah Mohd Razali, and Husni Hayati Mohd Rafdi

Behavioural Response of Cabbage White Butterfly (*Pieris rapae*) as Affected by Different Levels of Deltamethrin Insecticide 519
Jackie Lou F. Bagote, Jenny S. Palor, Angelo DG. Rullepa, Mark Argyll D. Tambaoan, and Schereid Joi N. Ugnasi

Storage Reserve Composition and Storage Behaviour of *Amorphophallus muelleri* Blume Seed 537
Siti Fadhilah, Eny Widajati, Satriyas Ilyas, Endah Retno Palupi, and Abdul Qadir

Synergistic Application of Marine-derived Actinomycetes and Microalgae to Enhance Rice Seedling Growth and Salinity Stress Tolerance 555
Alimuddin Ali, Muhammad Junda, Nur Haedar, Hasliana, Didik Imam Sakirin, and Herlina Rante

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