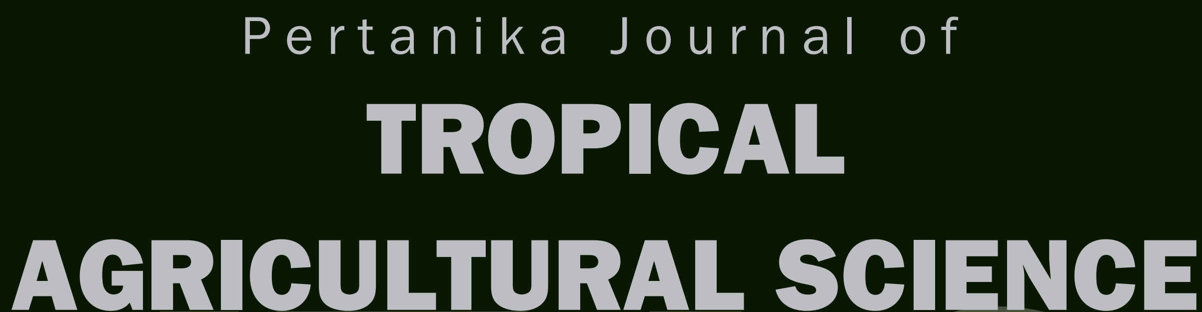
Pertanika Journal of

# 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 to improving quality in issues pertaining to tropical agriculture and its related fields.

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

The journal is available world-wide.

### *Aims and scope*

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

### *History*

*Pertanika* was founded in 1978. A decision was made in 1992 to streamline *Pertanika* into 3 journals as Pertanika Journal of Tropical Agricultural Science, Pertanika Journal of Science & Technology, and Pertanika Journal of Social Sciences & Humanities to meet the need for specialised journals in areas of study aligned with the interdisciplinary strengths of the university.

Currently, as an interdisciplinary journal of agriculture, the revamped journal, a leading agricultural journal in Malaysia now focuses on tropical agricultural research and its related fields.

### *Vision*

To publish journals of international repute.

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

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

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Most scientific papers are prepared according to a format called IMRAD. The term represents the first letters of the words ***I****ntroduction*, ***M****aterials and Methods*, ***R****esults*, ***A****nd* ***D****iscussion*. IMRAD is simply a more 'defined' version of the "IBC" (*Introduction*, *Body*, *Conclusion*) format used for all academic writing. IMRAD indicates a pattern or format rather than a complete list of headings or components of research papers; the missing parts of a paper are: *Title*, *Authors*, *Keywords*, *Abstract*, *Conclusions*, *References*, and *Acknowledgement*. Additionally, some papers include *Appendices*.

The *Introduction* explains the scope and objective of the study in the light of current knowledge on the subject; the *Materials and Methods* describes how the study was conducted; the *Results* section reports what was found in the study; and the *Discussion* section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the journal's **Instruction to Authors** (http://www.pertanika.upm.edu.my/Resources/regular_issues/Regular_Issues_Instructions_to_ Authors.pdf).

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

As articles are double-blind reviewed, material that may identify authorship of the paper should be placed only on page 2 as described in the first-4-page format in *Pertanika*'s **Instruction to Authors** (http://www.pertanika.upm.edu.my/Resources/regular_issues/Regular_Issues_Instructions_to_Authors.pdf).

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

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

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

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**Pertanika Journal of Tropical Agricultural Science**
**Vol. 45 (4) Nov. 2022**

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# Foreword

Welcome to the 4th issue of 2022 for the Pertanika Journal of Tropical Agricultural Science (PJTAS)!

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

This issue contains 18 articles; two review articles and the rest are regular articles. The authors of these articles come from different countries namely Australia, Bangladesh, Hungary, Indonesia, Malaysia, Nigeria, Pakistan, Singapore, Sri Lanka, and Thailand.

A regular article entitled "Isolation, Characterization, and Optimization of Keratinase from *Bacillus cereus* BRAW_KM" aimed to isolate, characterize, and optimize keratinase from *Bacillus cereus* BRAW_KM. The properties of keratinase were investigated using native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE. In addition, the ideal conditions of keratinase were adjusted by temperature, pH, and incubation time on enzyme activity. It resulted in the molecular weights of keratinase being 130 kDa and 95 kDa. Besides that, the best conditions were 29 °C, pH 9, and 90 minutes of incubation. The detailed information of this article is available on page 961

Ameera Abdul Reeza and her teammate from Universiti Teknologi MARA investigated the effect of organic waste fertilizers on growth and development of okra (*Abelmoschus esculentus*). The experiment was carried out in a randomized complete block design (RCBD) with 4 replications consisting of 5 treatments in duration of 6 weeks. The plant height, number of leaves, chlorophyll content, number of fruits, fresh and dry weight, and soil pH were the assessed parameters. The y found out that the NPK 12:12:17:2 (10 g) + chicken manure (25 g) might be the most suitable fertilizer combination to promote the higher growth of okra while reducing the dependency on inorganic compound fertilizers. Full information of this study is presented on page 1021.

A selected article entitled "A Review: Hormone Application for Artificial Breeding towards Sustainable Aquaculture" discussed the current research on artificial breeding in various fish species as well as new approaches or techniques to be applied to regulate the reproductive process in captive fish for sustainable aquaculture. The results show that the artificial hormones could offer a promising technique to breed fish in captivity, ensuring seed availability, improving genetic loss, and reducing the dependency on wild-caught fingerlings. The further details of this study are found on page 1035.

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.

In the last 12 months, of all the manuscripts processed, 39% were accepted. This seems to be the trend in PJTAS.

We would also like to express our gratitude to all the contributors, namely the authors, reviewers, Editor-in-Chief and Editorial Board Members of PJTAS, who have made this issue possible. PJTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

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TROPICAL AGRICULTURAL SCIENCE

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# Surgical Implantation of Acoustic Transmitters in *Neolissochilus soroides* and *Channa lucius* and Post-Surgical Wound Observation to Study Fish Telemetry

**Shazana Sharir[1,2], Nurfatin Zulkipli[2,6], Azhari Mohamad[2], Farah Ayuni Farinordin[3], Shafiq Zakeyuddin[4], Abdullah Samat[2], Amir Shah Ruddin Md. Sah[5] and Shukor Md Nor[2]***

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## ABSTRACT

Telemetry is an emerging method to study fish ecology and a reliable tool that provides useful information for managing and conserving aquatic fauna and river habitats. However, the reliability of telemetry depends on several technical factors. Implantation of the acoustic transmitter is one of the major aspects that ensure the survivability of the animal subject when released into the wild. Studies on the technicalities involving telemetry methods are limited; therefore, this study investigated the surgical insertion of an acoustic transmitter into the peritoneal cavity of *Channa lucius* and *Neolissochilus soroides* or locally known as Bujuk and Tengas. A severity index was used to rate the appearance of surgical wounds observed on the day of release into the river. Fish mortality and complications such as bleeding were not observed in both species post-surgery. The progress and prognosis of wound healing of *C. lucius* were better compared to *N. soroides*, with generally lesser inflammation and more sutures shed. Despite visually severe inflammation on *N. soroides* on day-12 and day-19 post-surgery, there was a good indication that

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skin integuments were healing. This data suggests that *C. lucius* has faster wound healing abilities compared to *N. soroides*. All fishes implanted with the acoustic telemetry method survived up to 244 days upon release into the Tembat River, Hulu Terengganu, Malaysia, and retained the tag throughout the tracking period.

*Keywords*: Acoustic telemetry, Bujuk, fish surgery, fish telemetry, Hulu Terengganu, Tengas, wound healing

## INTRODUCTION

The telemetry study provides precise information on fish movement behaviour and migrations by providing reliable real-time data compared to the conventional method of estimation using the mark-recapture technique (Dudgeon et al., 2015). Aquatic telemetry involves placing a transmitter (an electronic device) on or in the aquatic animal, which autonomously transmits data to data receiver stations (Whoriskey et al., 2019). However, the integrity of information depends on the quality and precision of data obtained (Brownscombe et al., 2019). Conducting a fish telemetry study involves considerations of various crucial parameters that could influence data interpretability. For example, fish survivability post-surgery influences the tracking period and swimming ability in its natural habitat. Thus, surgical skills, post-surgery care, and fish husbandry are important technicalities that indirectly influence the study's outcome (Ammann, 2020; Klinard et al., 2017).

Implantation of telemetry transmitters includes gastric and ovipositor insertion, as well as the external attachment. The most common technique is surgical implantation into the peritoneal cavity of the fish (Crossin et al., 2017) and is reputable for longer retention of the transmitter (Robinson et al., 2021). Nevertheless, peritoneal surgical insertions are comparatively invasive, requiring complex surgical procedures with prolonged duration, and are to be done by a surgeon with sedation (Lopes et al., 2016). Therefore, implantation of a transmitter into the peritoneal cavity of a fish can pose lethal and sub-lethal effects if conducted without experience or appropriate training (Cooke et al., 2013).

The principal premise of tagging procedures in telemetry studies is that tag implantation and device burden do not negatively influence the tagged animals' health, condition, behaviour, or survivability (Vollset et al., 2020). Thus, the increased practice of surgical fish tagging requires a complete understanding of tagging effects (Lopes et al., 2016; Wilson et al., 2017). Currently, most studies in the area are conducted in temperate countries such as Canada, the United States, and several European countries due to their technological advancement in fishery research using telemetry (Klinard et al., 2018; Thiem et al., 2011). However, similar studies are lacking in the tropical region, creating a lack of information on the application of the surgical approach and its recovery process in the local fish species and climate (Mitamura et al., 2006). Furthermore, available literature reviews tag implantation procedures that are species-specific which may vary in shape and size of the implants.

To date, a single study within the local region reports the use of two types of transmitters internally tagged in Mekong Giant Catfish or *Pangasius* sp. (Mitamura et al., 2006). The study was conducted on fishes of the Mekong River, which reported retention of the transmitter and absence of fungal infections post-procedure. Another informative study was conducted by Wagner et al. (2000), which looked at the effect of suture type on the wound healing of rainbows trout (*Oncorhynchus mykiss*). However, differences in climate and species do not favour direct referencing of the data from this study.

Malaysia is a nation with ambient temperature and high humidity and is home to various fish species. These factors may influence the surgical procedure and healing of the surgical wounds on the fish. Therefore, transparent recording of methods will enable future researchers to undertake a complete and informed procedure for a given telemetry study.

In addition to the surgical implantation technique, the predatory (*Channa lucius*) and benthopelagic (*Neolissochilus soroides*) species of fish were selected to study migration behaviour and habitat preferences, respectively (Harrington et al., 2022; Ullah et al., 2022). The *Channa* species have been reported to contain essential amino acids such as glycine, arachidonic acids, and polyunsaturated fats, which help promote wound healing (Kwan & Ismail, 2021). As a result, *C. lucius* may have a faster wound healing rate compared to *N. soroides*.

Proper healing of the surgical wounds on tagged fishes is crucial for their health and tag retention (Jepsen et al., 2002), and this will determine not only the success and interpretability of the study but also prevent financial loss. This study's single V9 VEMCO acoustic tag (Canada) costs approximately MYR 1,146.

This study aims to record the surgical insertion procedure of transmitters in two Malaysian freshwater fish species: *Neolissochilus soroides* and *Channa lucius*. This study also aims to qualitatively assess the healing of surgical wounds inflicted on *C. lucius* and *N. soroides* during the procedure. Wound healing was measured using photographic observation before the fish were released back into the river for telemetry study. Knowledge obtained from this study could aid future telemetry research, especially in the management of post-surgery procedures and research methods.

## MATERIALS AND METHODS

Fish subjects used in this telemetry study were native species of the Tembat River, Hulu Terengganu, Malaysia. Using native species reduces the unfamiliarity with the habitat in which the fishes will be released. Five (5) *N. soroides* fishes were caught using a baited fish trap, and the same number of *C. lucius* were caught using a baited hook and line. The fish subjects were then separately kept in a fibreglass tank with the environment mimicking their preferred habitat. The subjects were acclimatised for at least five days to ensure good health.

Stress response, fitness, and health during acclimatisation were observed based on normal and upright swimming behaviour as well as normal opercular movement (Chopin et al., 1996).

### Anaesthesia

In preparation for surgery, a 0.25 ml/L clove oil bath was first prepared in a 20 L transparent aquarium. The induction of anaesthesia was observed until stage 5 based on the sedation indication by Keene et al. (1998). A stage 5 of full sedation is indicated when the fish is in a recumbent position in the clove oil bath. The anaesthetised fish was carefully handled for measurement of length and weight prior to surgery.

Fishes were then positioned on a tray fitted with a V-shaped acrylic sheet upholstery to hold the fish upright dorsoventrally (Figure 1). The gills were constantly irrigated to maintain sedation throughout the procedure using a siphon and clove oil bath. The system consisted of a submersible pump placed in the anaesthesia bath that recycles the effluent onto the gills through a small tube (siphon) (Figure 1). The anaesthesia procedure is a modified method recommended by Matin et al. (2009) as well as Neiffer and Stamper (2009).

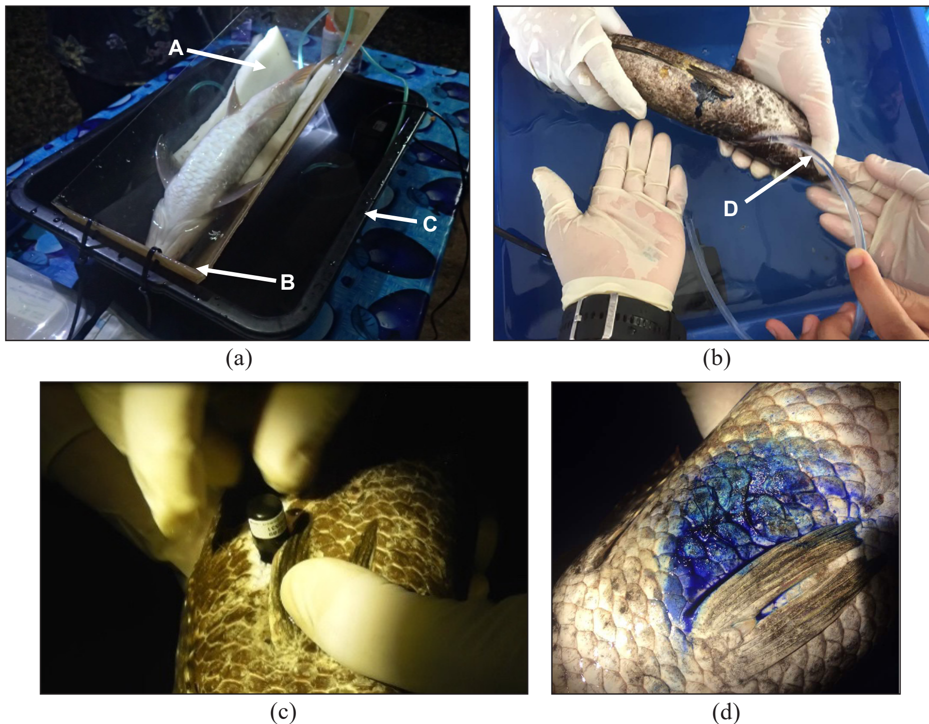



*Figure 1*. (a) The standard setting of placing a fish in a recumbent position on a sponge-based upholstery (A) in a triangular prism, (B) tray filled with sedative solution, (C) tilted prism to submerge fish's head under water, while the body remained above water for the surgery to be performed septically; (b) Clove oil anaesthetic solution was used to directly irrigate the gills using a siphon hose (D) powered by an aquarium-grade motor pump; (c) The tag was snugly inserted into the peritoneal cavity; (d) The wound was topically treated with an antiseptic solution to prevent infection and promote wound healing

## Surgical Implantation of Acoustic Tag

Implantation of both fish species was conducted using different surgical sites based on the unique anatomy of *N. soroides* and *C. lucius*, as reported by Rożyński et al. (2017) and Schoonyan et al. (2017).

The procedure was started with the removal of scales using forceps. Approximately15-20 pieces of scales were removed for *C. lucius* as this species has finer-sized scales. In addition, scales were removed as individual scales have a high resistance to piercing, and the overlapping position of the scales would collectively distribute the puncture over a bigger area (Vernerey et al., 2014).

On *C. lucius*, the incision was made between the posterior of the pectoral fin and the anterior of the anus, where the peritoneal cavity is located (Figure 2). As for *N. soroides*, the incision was made parallel to the midline of the ventral side of the fish between the pectoral and anal fins (Figure 3). A fine incision measuring 3.5 ± 0.12 cm was first made using a surgical blade, followed by turning on the tag using a VEMCO 180kHz Tag Activator (VTA-180k-V9, Canada) and insertion of the acoustic tag into the peritoneal cavity. The V9 VEMCO (Canada) transmitter dimension was 9 × 29 mm and weighed approximately

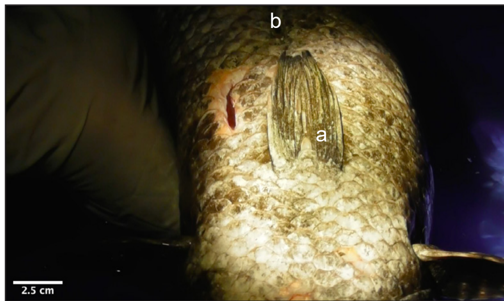



*Figure 2*. The incision made on *Channa lucius* was located between the pelvic fin (a) and anus (b)

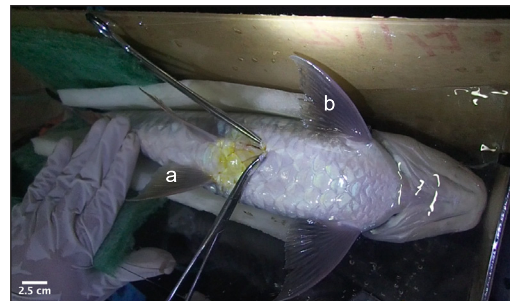
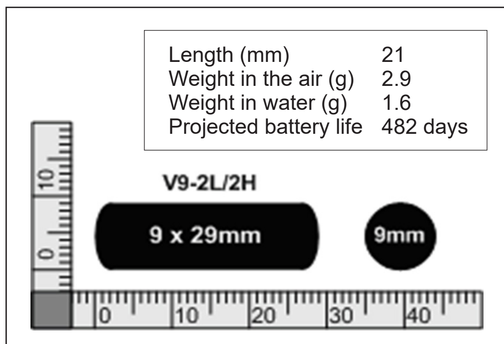



*Figure 3*. The incision made on *Neolissochilus soroides* was located between the pelvic fin (a) and pectoral fin (b)




(a)

(b)

*Figure 4*. (a) Schematic dimension of each V9 VEMCO acoustic transmitter used in this study; (b) the V9-180khz acoustic transmitter with a tag activator in the background

2.9 g (Figure 4). The incision was closed with three simple interrupted sutures and finished with a surgeon knot without gaping. A topical antibiotic: Biobandage (Hikari, USA), was applied to the wound to prevent secondary infection and promote healing.

### Recovery

Immediately after suturing, the fish was transferred to the recovery aquarium for stabilisation and observed until stage 5 of recovery (Keene et al., 1998). At stage 5 of anaesthetic recovery, the fish displayed total behavioural recovery with normal swimming movement. The highly aerated recovery bath was maintained at 24–25 °C to minimise stress and ensure a quick recovery. Injury or fatality was also prevented by securely covering the tanks to avoid fish from escaping. Fishes were kept in these tanks for a minimum of seven days, during which their wound healing was monitored before release into the Tembat River.

### Observation of Wound

The wound on each fish was observed after surgery and once on the day of release (between 13-82 days). Repeated examination of the wounds was not conducted to avoid inducing stress. Instead, the wounds were inspected for the number of sutures retained and assigned with an index representing severity value (Adams et al., 1993; Paukert et al., 2001) (Table 1). Adams et al. (1993) suggested the following indexing based on selected parameters:

Maximum wound gape, whereby no gape was scored as 0, <1.0 mm gape as 10, and a gape of >1.0mm as 20.

Wound redness, whereby no redness was scored as 0, redness only near incision and sutures as 10, redness that had expanded beyond sutures as 20, and redness at the entire area around sutures as 30.

Other physiological or physical wound changes were recorded, such as the production of excess mucous on the skin. Consistency of indexing was maintained by assigning the same observer to indicate scores for all fish subjects.

## RESULTS

All *C. lucius* and *N. soroides* observed in this study survived the procedure without signs of inflammation and bleeding after surgery. Within 24 hours post-surgery, fish also did not show lethargy or erratic swimming. However, various levels of wound healing

Table 1
*Severity index based on the appearance of the surgical wounds of* N. soroides *and* C. lucius

| Observed parameter | Severity Index |
|---|---|
| | No gape = 0 |
| Wound gape | Gape < 1.0 mm = 10 |
| | Gape > 1.0 mm = 20 |
| | No redness = 0 |
| Inflammation (redness) | Only near incision and suture = 20 |
| | The entire area around incision = 30 |

progress were observed from day 13 to day 82, ranging from moderate inflammation, shedding of sutures, and excess mucous production.

The progress of post-surgery wound healing on *C. lucius* between the 1st, 13th, 36th, and 82nd days after surgery were photographically recorded. On the first day post-surgery, the wound typically showed no complications with a clear and clean-cut through the muscle without bleeding. Figures 5 (a), (b), and (c) show the wound progress observed before release on day 13 for subjects ID 16289 and ID 16330, while ID 16312 had shedding of at least two sutures. Although the severity of inflammation was different between subjects, muscle adhesion progressed well without gaping.

However, subject ID 16312's incision wound was moderately inflamed with all sutures shed. Gaping of the wound was also observed at the posterior end of the incision (Figure 5 (b)). Figure 5 (d) shows wound healing progress observed for subject ID16297 on day 36, whereby one suture was shed and slight inflammation was visible. Figure 5 (e) shows the wound healing progress on day 82 for subject ID 16285. Two sutures were shed without severe inflammation and gaping, but mild inflammation was noted in the area where scales were extracted.

The progress of wound healing in *N. soroides* was observed on the 12th, 13th, and 19th days post-surgery (Figure 6). No complications or bleeding were observed during the surgery, but mild inflammation was seen where the scales were removed. Three sutures were applied to close the surgical wound gap in *N. Soroides*.

Wound incisions on subjects with ID 16342, ID 16174, and ID 16170 were observed on day 12 and 13 post-surgery, respectively (Figure 6 (a), (b), and (c)). These subjects displayed moderate inflammation of the surgical wound while all initial sutures remained intact. The moderate inflammation was accompanied by excess mucous around the suture and where scales were extracted. The surgical wounds of ID 16090 and ID 16089 were observed on day 19 post-surgery. For ID 16090, one suture was shed, and inflammation was observed only at the

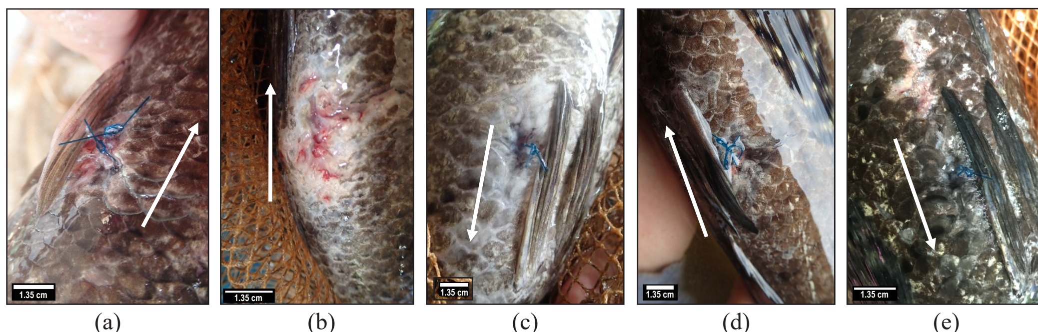
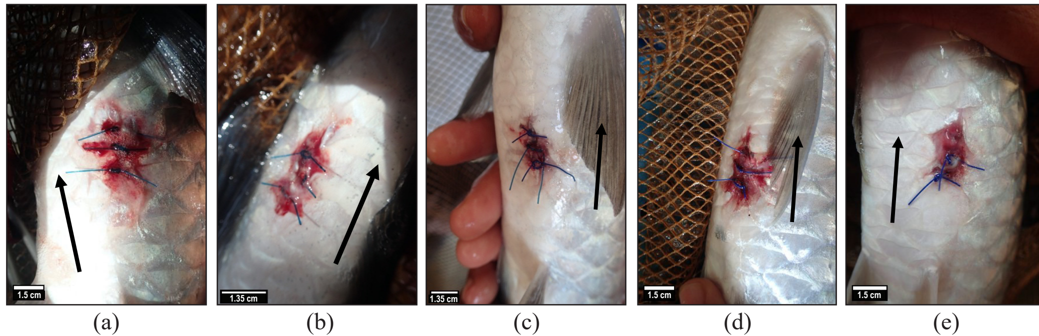



*Figure 5*. The appearance of the surgical wounds of selected *Channa lucius* was recorded on the day of release. The arrow in the photos indicates the direction of the head: (a) Day 13 – ID 16289; (b) Day 13 – ID 16312; (c) Day 13 – ID 16330; (d) Day 36 – ID 16297; and (e) Day 82 – ID 16285




(a) (b) (c) (d) (e)

*Figure 6*. The appearance of the surgical wounds of selected *Neolissochilus soroides* was recorded on the day of release. The arrow in the photos indicates the direction of the head: (a) Day 12 – ID 16342; (b) Day 12 – ID 16174; (c) Day 13 - ID 16170; (d) Day 19 – ID 16089; and (e) Day 19 – ID 16090

incision area. Moreover, ID 16089 had all its original sutures intact with one loose suture, but inflammation was observed at the incision site and underneath the scale layers. Excess mucous was also observed on the wound, pectoral fin, and body.

Based on the Severity Index by Adams et al. (1993), the number of sutures retained was higher in *N. soroides* compared to *C. lucius*, while inflammation was more severe in *N. soroides* compared to *C. lucius* (Table 2). As Adams (1993) suggested, fish with higher severity index show lesser progress in wound healing. It was visually comparable, whereby *N. soroides* had inflammation (redness of the wound) extended beyond the incision, while *C. lucius* had very little to no inflammation.

## DISCUSSION

Both fish species used in this study have scales as their integument anatomy, particularly of the cycloid type (Farinordin et al., 2017). However, both species differ

Table 2
*The severity index assigned to each fish based on observation after 12 to 83 days of surgery for* N. soroides *and* C. lucius

| Species | Fish ID | Day observed | Total length (cm) | Total weight (g) | No. of suture retained | Wound gape | Inflammation |
|---|---|---|---|---|---|---|---|
| *Channa lucius* | ID 16289 | 13 | 43 | 843 | 2 | 0 | 20 |
| | ID 16297 | 36 | 41 | 640 | 2 | 0 | 20 |
| | ID 16330 | 13 | 53 | 1,662 | 1 | 0 | 0 |
| | ID 16285 | 82 | 43 | 745 | 1 | 0 | 0 |
| | ID 16312 | 13 | 43 | 713 | 0 | 0 | 20 |
| *Neolissochilus soroides* | ID 16170 | 13 | 37.5 | 550 | 3 | 0 | 20 |
| | ID 16090 | 19 | 35 | 659 | 2 | 0 | 20 |
| | ID 16089 | 19 | 40 | 580 | 3 | 0 | 30 |
| | ID 16342 | 12 | 41.5 | 665 | 3 | 0 | 30 |
| | ID 16174 | 12 | 43 | 731 | 3 | 0 | 30 |

in the size of their scale, which influenced the number of scales extracted for the surgical procedure. For example, *C. lucius* scales are small (0.2-0.3 cm) and densely overlapping, requiring the removal of more scales. Meanwhile, *N. soroides* only required two to three larger soft scales (1-2.5 cm) to be extracted to perform the surgical implantation.

Although scale removal only creates a surface wound, a deeper cut of the muscle tissue required to complete the procedure causes bleeding (Sveen et al., 2020). However, during the incision, minimal bleeding was observed, which is due to blood vessel constriction stimulated by the proinflammatory cytokines. This mechanism happens within a few minutes of wound formation and helps prevent excessive blood loss (Schmidt et al., 2016). Since it was time-consuming to make an incision on *C. lucius* as the scales were small, structurally tough, and dense, the subject must be fully sedated to lessen the stress of the procedure. Comparatively, incisions on *N. soroides* were made much quicker as the fish scales were larger, softer, and pliable, which made extracting the scales easier. It was also observed that *C. lucius* took longer to be fully sedated and recover compared to *N. soroides*. It is due to the ability of *C. lucius* to breathe air while *N. soroides* lack in that capability.

Furthermore, prolonged exposure to stress could delay the wound healing process (Sveen, 2018); thus, the fish were not repeatedly handled for wound monitoring between post-surgery and release. Normally, a surgical wound or a deep wound in teleost will activate a series of healing processes, which includes: re-epithelialisation, inflammation, granulation tissue formation, and tissue remodelling (Sveen et al., 2019). During the process, leukocytes and macrophages are recruited to clear the wound from damaged tissue and drive the repair processes (Richardson et al., 2013). Inflammation is a response to re-epithelialisation and is supposedly an indicator of wound healing (Sveen et al., 2020). Inflammation in adult fish usually starts on day 1 post-surgery to day 65, with marked redness on day 5 to 10 (Schoonyan et al., 2017). Based on the severity index used in this study, most *C. lucius* showed slight inflammatory reaction or redness around the incision area. All *N. soroides* fish showed prominent inflammatory reactions even though the observation was made after 12 days of surgical procedure.

Subjects ID 16090 (*N. soroides*) and ID 16312 (*C. lucius*) exhibited the formation of granulation or repair tissue. Granulation tissue usually grows from the wound's borders and replaces the damaged tissue with time. Granulation tissues contain connective tissue, fibroblasts, myofibroblasts, immune cells, and small blood vessels (Roubal & Bullock, 1988). In fish ID 16090, granulation tissues grew at infringement of the suture. For subject ID 16312, all sutures were shed, and granulation tissue was seen to grow in an unorganised manner. The growth of granulation tissue seen in this study was consistent with data from the literature, particularly Fontenot and Neiffer (2004),

Schmidt et al. (2016), and Sveen et al. (2019), whereby granulation occurred from the 6th day to the 75th day post wounding.

Moreover, the complete scale regeneration took place within one month after wounds were inflicted (Richardson et al., 2013). In this study, fish ID 16330 had almost fully regenerated skin, but scales were not regenerated in 13 days. Darker pigmentation was additionally observed on *C. lucius*, ID 16330, which may have been caused by the rapid release of melanin granules, causing pigmentation of the skin around the wound (Rai et al., 2012).

Additionally, all fish had excessive mucous formation on the skin and the wound, which is typical in a wounded fish (Sveen et al., 2020). The mucous gel produced by wounded fish provides physical and antimicrobial protection to the wound surface. The secretion may also enhance wound healing through haemolytic activity and promote vasoconstriction of smooth muscle cells (Akunne et al., 2016).

Another notable observation was the different rate of suture shedding between both species. Shedding of suture occurred on all *C. lucius* and one out of five *N. soroides*. Suture shedding is normal and occurs in other species within 20 days post-surgery (Cooke et al., 2003; Schoonyan et al., 2017) and is also an indicator of healing. In this study, *C. lucius* retained a lesser number of sutures compared to *N. soroides*, while *N. soroides* had significantly more inflammation compared to *C. lucius*. This study also shows, *C. lucius* have a faster wound healing compared to *N. soroides* based on the sutures shed and the inflammation. According to Sveen et al. (2020), the healing of deep wounds is species-dependent, meaning that some species have a much faster healing rate than others. In this case, the faster healing by *C. lucius* may be attributed to the findings by Sahid et al. (2018), in which the study found an active compound produced by channids species that can help accelerate wound healing.

Proper healing of surgical wounds can take up to 100 days, depending on several factors, such as species and local climate (Sveen et al., 2020). Accommodating prolonged timeline was impractical for a telemetry study, while releasing fishes without sufficient recovery may pose a risk of losing the transmitter. Furthermore, losing the transmitter would generate biased data on movement and mortality; a key parameter used in telemetry studies. Therefore, based on the observation in this study, the reasonable but effective duration of time to confine is between 4 to 13 days after surgery for *C. lucius* and 15 to 17 days after surgery for *N. soroides.*

According to Schoonyan et al. (2017), incision closure, inflammation, and the presence of sutures are common indicators of healing rate; thus, it is very important to observe these attributes to ensure fish health post-surgery. In this observation, all subjects showed indications of recovery through shedding sutures, tissue inflammation, and excellent movement agility. Thus, the fish were considered fit and released back into the river for movement and habitat study

(Paukert et al., 2001). The continuity of this study was published in Sharir et al. (2021), whereby all of the fishes were reported to survive and had tag retention for up to 244 days.

## CONCLUSION

The assessment of wound severity is an indicator of fish health, and both species in this study responded differently to the surgical procedure. *Channa lucius* showed signs of recovery between 13 to 82 days post-surgery with lesser inflammation and a lesser number of intact sutures compared to *N. soroides*. Although most *N. soroides* demonstrated severe inflammation, survivability was not affected. Nevertheless, both species exhibited a high level of fitness with high agility in swimming behaviour before being released. Based on the current data, surgical tagging of fish is recommended for acoustic telemetry study with retention of the fish in a recovery tank for at least 13 days before being released. This procedure would help ensure appropriate wound healing and survivability in the wild. This duration is also recommended to maximise battery power utilisation of the tags.

The study indicated that the peritoneal insertion of the VEMCO V9 acoustic tag is suitable for *C. lucius* and *N. soroides*. In addition, it may be applicable for other local fishes with similar size and body forms, such as the snakehead species (*Channa micropeltes* and *Channa striata*) and fusiform species (such as *Tor* sp. and *Hampala macrolepidota*). Furthermore, the surgical procedure is proven safe and efficient with retention of the tags, health maintenance, and no reported mortality.

## ACKNOWLEDGEMENTS

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# Substituting *Sargassum* sp. Compost for Inorganic Fertilizer Improves the Growth and Yield of Shallot (*Allium cepa* L. Aggregatum Group)

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## ABSTRACT

Fertilizers derived from natural materials, such as *Sargassum* sp. (seaweed), are a promising technique to overcome the negative impact of overuse of inorganic fertilizers. Groundwater contamination, soil degradation, and changes in the soil microorganism community are problems related to overdosing on inorganic fertilizer during crop production. The use of *Sargassum* compost (SC) as a substitute inorganic fertilizer was tested by evaluating the growth and yield of shallot grown on sandy soil. The research was arranged in a randomized complete block design consisting of four treatments and three blocks of replications. The treatments involved substituting SC for inorganic fertilizer, which were 100% inorganic, 25% SC + 75% inorganic, 50% SC + 50% inorganic, and 75% SC + 25% inorganic, respectively. The compositions of nitrogen (N), phosphorus (P), potassium (K), sodium (Na), sulfur (S), auxin, gibberellin, cytokinin, and kinetin in SC, as well as the growth and yield of shallot, were analyzed by analysis of variance followed by the least significant difference test. The results showed that the SC contained high organic matter (45.78%), nitrogen (4.1%), phosphate (0.5%), potassium (0.8%), sodium (7.2%), sulfur (0.2%), and plant growth hormones, such as auxin (8.14 $mg.g^{-1}$), gibberellin (15.97 $mg.g^{-1}$), cytokinin (7.70 $mg.g^{-1}$), and kinetin (2.78 $mg.g^{-1}$). Interestingly, all substitution levels of the SC for inorganic fertilizer improved nutrient absorption in

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the leaves, roots, and bulbs. Moreover, the growth and yield of shallot were not significantly different among the treatments. Therefore, to provide sufficient nutrients and growth hormones, SC could be substituted for up to 75% of organic fertilizers for shallot plants.

*Keywords*: Growth hormones, nitrogen, phosphorus, potassium, sandy soil

## INTRODUCTION

Indonesia has a biodiversity of seaweeds and *Sargassum* sp. is one of the most abundant seaweeds living in the Indonesian seas (Widyartini et al., 2017). This seaweed is a brown alga with a land plant-like morphology in threads or sheets that lives in clear water or choppy and rocky areas and thrives at a depth of 0.5−10 m (Muslimin & Sari, 2017). *Sargassum* sp. contains many organic ingredients, hormones, and amino acids (Silva et al., 2019). Isnansetyo et al. (2017) extracted *Sargassum* brown seaweed to produce fucoidan as an anti-cancer ingredient. The extraction process produced 40% of seaweed waste from the dry material used (Flórez-Fernández et al., 2021). Silva et al. (2019) reported that *Sargassum* contained N 17.4%, P 4.5%, and K 7.2%. Sembera et al. (2018) reported that *Sargassum* as compost contained organic matter of 36.3%, a ratio of carbon to nitrogen (C/N ratio) of 13.5, and a pH of 8.4. Therefore, the *Sargassum* waste could contain high organic matter, potentially as organic fertilizer. We make *Sargassum* compost (SC) from *Sargassum* waste with *Bacillus* sp. decomposition. The SC was analyzed and found that it contained organic matter of 45.78%, a C/N ratio of 11.17, and a pH of 7 (Table 1).

Organic fertilizer is a sustainable choice for nutrient and organic matter input (Chatterjee et al., 2017; Renuka et al., 2016). Other advantages of organic fertilizers are that they increase soil fertility, increase organic matter, improve soil structure, increase moisture content, increase cation exchange capacity, increase nutrient availability, and release nutrients more slowly and more consistently (Roba, 2018). In addition, organic matter will increase soil microorganisms' activity, affecting the availability of nutrients (Amujoyegbe et al., 2007). Elumalai and Rengasamy (2012) reported decomposition of *Sargassum* with *Bacillus* in contained 0.06 mg/g nitrogen dioxide ($NO_2$), 14 mg/g nitrate ($NO_3$), 1.71 mg/g phosphate ($PO_4$), and 8 mg/g potassium (K).

*Sargassum* waste as an organic fertilizer for shallot (*Allium cepa* L. Aggregatum group) has not been reported in Indonesia. Shallot is an important crop in tropical countries, including Indonesia, as it is useful as a food seasoning and traditional medicine and has high commercial value (Sulistyaningsih et al., 2020). The popularity of shallot has increased yearly consumption (Ministry of Agriculture [MOA], 2019). Consequently, the shallot is cultivated intensively with harvests two or three times per year and extensively in many areas, including sandy coastal areas.

The intensive cultivation of shallot with inorganic fertilizers has the potential to pollute the environment. Shallot cultivation

Table 1
*Properties (mineral type, unit and value) contained in the* Sargassum *compost, method and equipment used for analysis*

| Mineral type | Unit | Value | Method | Equipment |
|---|---|---|---|---|
| N | % | 4.1 | Wet destruction | UDK 139 Semi-Automatic Kjeldahl Distillation Unit (Velp Scientifica, Italy) |
| P | % | 0.5 | Wet destruction | GENESYS™ 10 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) |
| K | % | 0.8 | Wet destruction | Flame photometer PFP7C (Rose Scientific. Ltd., Canada) |
| Na | % | 7.2 | Wet destruction | Flame photometer PFP7C (Rose Scientific. Ltd., Canada) |
| S | % | 0.2 | Wet destruction | GENESYS™ 10 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) |
| Auxin | $mg.g^{-1}$ | 8.14 | Linskens and Jackson (1987) | High-performance liquid chromatography (HPLC) (Thermo Fisher Scientific, USA) |
| Gibberellin | $mg.g^{-1}$ | 15.97 | Linskens and Jackson (1987) | High-performance liquid chromatography (HPLC) (Thermo Fisher Scientific, USA) |
| Cytokinin | $mg.g^{-1}$ | 7.70 | Linskens and Jackson (1987) | High-performance liquid chromatography (HPLC) (Thermo Fisher Scientific, USA) |
| Kinetin | $mg.g^{-1}$ | 2.78 | Linskens and Jackson (1987) | High-performance liquid chromatography (HPLC) (Thermo Fisher Scientific, USA) |
| Water content | % | 2.26 | Gravimetric | Oven (Memmert, Germany) |
| C-organic | % | 45.78 | Dried combustion | Muffle furnace (MXBAOHENG, China) |
| C/N ratio | | 11.17 | Dried combustion | Muffle furnace (MXBAOHENG, China) |
| pH | | 7 | Potentiometrically | pH Meter (Hanna Instruments, Indonesia) |
| *Bacillus* sp. | $CFU.ml^{-1}$ | $10^8$ | Plate count | GENESYS™ 10 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) |

requires inorganic fertilizer, such as 102.6 $kg.ha^{-1}$ N, 89.8 $kg.ha^{-1}$ P, and 121 $kg.ha^{-1}$ K, as recommended by Minhal et al. (2019). The continuous overuse of inorganic fertilizers pollutes groundwater, increases air pollution, increases the weed population, and degrades the soil and microorganisms (Rahman & Zhang, 2018). Innovation is needed to provide nutrients for shallot plants to maintain soil health and environmental sustainability. The use of organic fertilizer from *Sargassum* seaweed could be a promising method for maintaining a healthy environment. Adding seaweed as an organic fertilizer supplies macro and micronutrients improves soil structure, increases water availability and soil cation exchange capacity, and increases plant resistance to biotic and abiotic stressors (de Siqueira Castro et al., 2017; Dineshkumar, 2020; Divya et al., 2015).

Therefore, this study was designed to investigate the potency of *Sargassum* waste compost as a substitute for inorganic fertilizer on the growth and yield of shallot planted in coastal sandy soil. Coastal sandy soil was used as the planting medium because it has high filtration capacity, low organic matter, and lacks nutrients (Šimanský et al., 2019).

## MATERIALS AND METHODS

### Plants and Material Preparation

The research was conducted from June 2021 to August 2021 at the Universitas Gadjah Mada (UGM) experimental station, Banguntapan, Bantul, Special Region of Yogyakarta Province, Indonesia (07°48’ 17”S and 110°24’ 45”E) at an altitude of 107 m above sea level. The mean temperature during the experiment was 29.5 °C, the humidity was 63.5%, and the sunlight intensity was 44,333 lux. The planting medium was coastal sandy soil with contents of N 0.01%, P 0.0016%, K 0.000032%, pH 6.55, moisture level 0.59%, organic matter 9.16%, cation exchange capacity (CEC) 5.43 $mg \cdot eq \cdot kg^{-1}$, and electrical conductivity (EC) 1.02 $dS \cdot m^{-1}$. The shallot variety was Bima Brebes. The seaweed material was *Sargassum* sp. waste from the fucoidan extraction process (Isnansetyo et al., 2017).

### *Sargassum* Compost Analysis

The *Sargassum* sp. waste was composted by fermentation for seven days after adding 3% molasses, 20% water, and 0.001% isolated *Bacillus* sp. The SC was analyzed N, P, S, K, Na, auxin, gibberellin, cytokinin, and kinetin contents. The N levels were analyzed using the wet destruction method and the UDK 139 semi-automatic Kjeldahl distillation unit (Velp Scientifica, Italy). Tissue P and S levels were determined using the wet destruction method with a GENESYS™ 10 UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). The K and Na levels were measured using the wet destruction method with a flame photometer PFP7C (Rose Scientific. Ltd., Canada) (Balittanah 2009). Auxin, gibberellin, cytokinin, and kinetin contents were determined using the method of Linskens and Jackson (1987) and high-performance liquid chromatography equipment. The data are listed in Table 1.

### Experimental Design

This experiment was set as a single factor randomized complete block design with three blocks as replications. There were four treatments with combinations of the inorganic fertilizer and the SC (Table 2). The 12 bulbs were planted in planting boxes (45 × 33 × 16 cm) with a 10 × 10 cm planting distance. The fertilizer was applied in three stages, such as at the time of bulb planting and at 28 and 42 days after planting.

Table 2
*The combinations of fertilization treatments*

| Treatment | Type of fertilizer ($kg.ha^{-1}$) | | | |
|---|---|---|---|---|
| | *Sargassum* sp. compost | Inorganic | | |
| | | N | P | K |
| 0% SC + 100% Inorganic | 0 | 102.6 | 89.8 | 121 |
| 25% SC + 75% Inorganic | 600.5 | 76.9 | 67.3 | 90.7 |
| 50% SC + 50% Inorganic | 1,201.0 | 51.3 | 44.9 | 60.5 |
| 75% SC + 25% Inorganic | 1,801.5 | 25.6 | 22.4 | 30.2 |

### Planting Medium Content Analysis

The planting media data consisted of soil organic matter content, bulk density ($g \cdot m^{-3}$), moisture content (%), EC ($dS \cdot m^{-1}$), and pH (Balittanah, 2009). The organic matter content was measured by ashing the sample in a muffle furnace at 550–600°C for 4 hours. The organic matter became carbon dioxide ($CO_2$), and the metal became a metal oxide. The weight of the missing material was the organic matter, which was converted to C-organic content after multiplying by a factor of 0.58. The bulk density ($g \cdot cm^{-3}$) was calculated by the weight of dry soil ($M_{solids}$) divided by the total soil volume ($V_{soil}$). The total soil volume was the combined volume of the solids and the pores, containing air ($V_{air}$), water ($V_{water}$), or both. The moisture content (%) was determined by calculating the weight of the field capacity soil divided by the weight of oven-dried soil at 105 °C for 4 hours multiplied by 100%. The EC and pH were measured by weighing 10 g of planting medium into a shaker bottle, adding 50 ml of distilled water, beating the mixture with a whisk for 30 min, and using an EC meter with sodium chloride (NaCl) calibration for the EC value. The pH was determined with a pH meter (Hanna Instruments, Indonesia).

### Shallot Tissue Analysis

The shallot tissue included N, P, S, K, and Na. The methods of tissue analysis were as follow in SC analysis.

### Growth and Yield of Shallot

Growth and yield were measured by plant height (cm), the number of leaves, leaf area ($cm^2$), total fresh weight (g), total dry weight (g), and fresh bulb weight (g). The leaf area was calculated with the WinDIAS 3 leaf area meter (United Kingdom). The total dry weight was calculated from total biomass dried in an oven at 80 °C for 48 hours.

### Statistical Analysis

All data obtained were analyzed for analysis of variance with a significance level of 0.95, followed by the least significant difference (LSD) test. This analysis was performed using *R* statistical software (v. 3.2.2) (The R Foundation for Statistical Computing, Austria).

## RESULTS AND DISCUSSION

### *Sargassum* Compost Content

The SC was analyzed before being applied as a planting medium. The SC was processed through fermentation for seven days after adding 3% molasses, 20% water, and 0.001% of a *Bacillus* sp. isolate. The properties of the SC and the analytical method are shown in Table 1. The proportion of C-organic and the C/N ratio of the SC were 45% and 11.17, respectively. According to the Ministry of Agriculture (MOA), Indonesia (2011), those values were standard characteristics of SC. The standard requirement for C-organic is >15, and the C/N ratio should range between 10 and 20. The C/N ratio indicates the decomposition process in which a lower ratio value indicates the higher availability

of nutrients (Setiawati et al., 2018). The pH value (7.0) and water content (2.26%) showed that the SC was available to be used as organic fertilizer. The contents of essential nutrients were 4.1% N, 0.5% P, 0.8% K, 7.2% Na, and 0.2% S, respectively. It also contains hormones to regulate plant growth. The hormones in the SC were 15.97 $mg \cdot g^{-1}$ gibberellin, 8.14 $mg \cdot g^{-1}$ auxin, 7.70 $mg \cdot g^{-1}$ cytokinin, and 2.78 $mg \cdot g^{-1}$ kinetin.

**Planting Medium Characters**

The sandy soil containing the combined SC and inorganic fertilizer treatments 65 days after planting showed that the organic matter and moisture contents increased (Table 3). The 75% SC + 25% inorganic fertilizer treatment had a significantly increased amount of organic matter (9.77%) and moisture content (9.86) compared to those in the 100% inorganic fertilizer treatment. The increase in organic matter content by SC improved the soil moisture content. *Sargassum* contains alginate, which absorbs and stores water. The results confirm that applying seaweed as an organic fertilizer could make soil store more water (Sinulingga & Darmanti, 2007). Other studies have reported that seaweed applications increase organic matter and absorption of water and minerals in the topsoil (Abdel-Raouf et al., 2012; Raghunandan et al., 2019). Soil moisture content can be used as a basis for measuring the availability of water in the planting medium. Water is a vital component of plant metabolism (Osakabe et al., 2014). Therefore, the quality of the sandy soil as a planting medium would improve based on the SC's organic matter and moisture contents.

**Tissue Analysis**

The highest N content among the treatments was in the leaves, followed by root and bulb. The highest N content value (3%) was found in the leaves with a combined treatment of 50% SC + 50% inorganic (Figure 1). SC substitution up to 50% did not affect the

Table 3
*Organic matter, bulk density, moisture content, and pH of the sandy soil after treatment with the* Sargassum *compost 65 days after planting*

| Treatment | Organic matter (%) | Bulk density ($g.cm^{-3}$) | Moisture content (%) | EC ($dS.m^{-1}$) | pH |
|---|---|---|---|---|---|
| 0% SC + 100% Inorganic | 9.64 ± 0.09b | 1.55 ± 0.01a | 9.41 ± 0.36b | 1.03 ± 0.01 b | 6.54 ± 0.03a |
| 25% SC + 75% Inorganic | 9.67 ± 0.04b | 1.56 ± 0.01a | 9.52 ± 0.14ab | 1.07 ± 0.00 ab | 6.54 ± 0.02a |
| 50% SC + 50% Inorganic | 9.70 ± 0.01ab | 1.57 ± 0.01a | 9.64 ± 0.15ab | 1.80 ± 0.00 a | 6.56 ± 0.01a |
| 75% SC + 25% Inorganic | 9.77 ± 0.01a | 1.57 ± 0.03a | 9.86 ± 0.09a | 1.90 ± 0.05 a | 6.56 ± 0.01a |
| CV (%) | 7.46 | 8.09 | 9.20 | 9.87 | 7.10 |

*Note*. Means followed by the same letters in the same column are not significantly different according to the LSD test; α = 5%

accumulation of leaf N levels but decreased at 75% SC substitution. The accumulation of nitrogen in the leaves shows that N plays a role in many photosynthetic processes. Plants require nitrogen for metabolism and protein syntheses, such as chlorophyll and cell division (Agirman & Cetin, 2015; Iqbal et al., 2020). After leaves, N content accumulates in the root. Substitution of 25% SC showed the highest N content in root and decreased at 50% to 75% SC. The N element that accumulates in the roots is useful in the root elongation process to make the roots more flexible in nutrient absorption. Applying seaweed also increases the activities of enzymes involved in the N cycle, such as ureases and dehydrogenases (Wang et al., 2018). The last application, 0% to 75% SC substitution, showed that N content in the bulb tended to be stable.

Figure 2 shows that the highest average P content was detected in the bulbs, except in the 25% SC + 75% inorganic treatment. By increasing the SC percentage for inorganic fertilizer, the P content in the bulb tended to increase, and the highest P content (0.44%) was found in the combined 75% SC + 25% inorganic treatment. Elemental P plays a role in assimilating transport, affecting the size and weight of onion bulbs (Anbes et al., 2018). It shows that the shallot wants P, which is always available, unlike the case with the root and leaves of shallot, which prefer P inorganic. It is evident that the smaller the inorganic fertilizer, the lower the P content in the roots and leaves.

K content accumulated the highest in the roots of all treatments (Figure 3). The K content increased as the percentage of SC applied in the planting medium was increased. Elemental K strengthened the cell walls of shallot, which supports the roots to penetrate the media and absorb water (Gunadi, 2009). After the root, K content accumulates in the bulb. K content in bulb showed stable results at 0.63 to 0.80%. However, it is different from the K content in shallot leaves; K content also increased as the percentage of SC applied in the planting medium was increased. Elemental

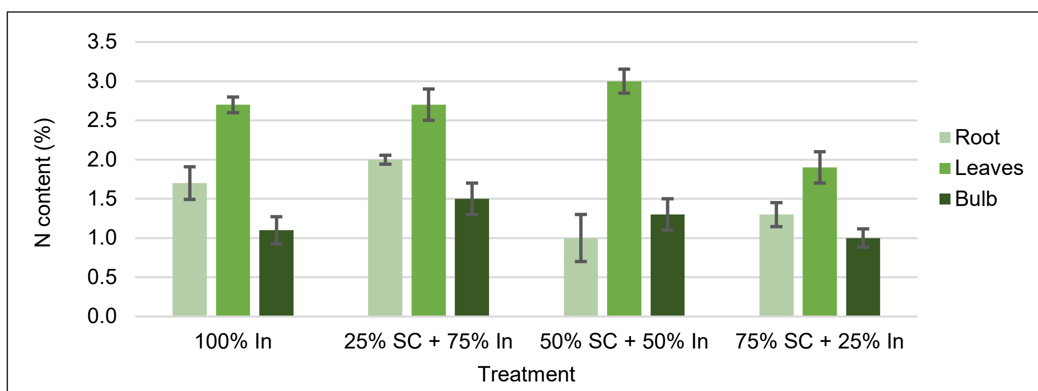


*Figure 1*. Tissue analysis of N content in roots, leaves, and bulbs with four treatments: 100% In = 100% inorganic fertilizer; 25% SC + 75% In = 25% *Sargassum* compost + 75% Inorganic fertilizer; 50% SC + 50% In = 50% *Sargassum* compost + 50% Inorganic fertilizer; and 75% SC + 25% In = 75% *Sargassum* compost + 25% Inorganic fertilizer, respectively

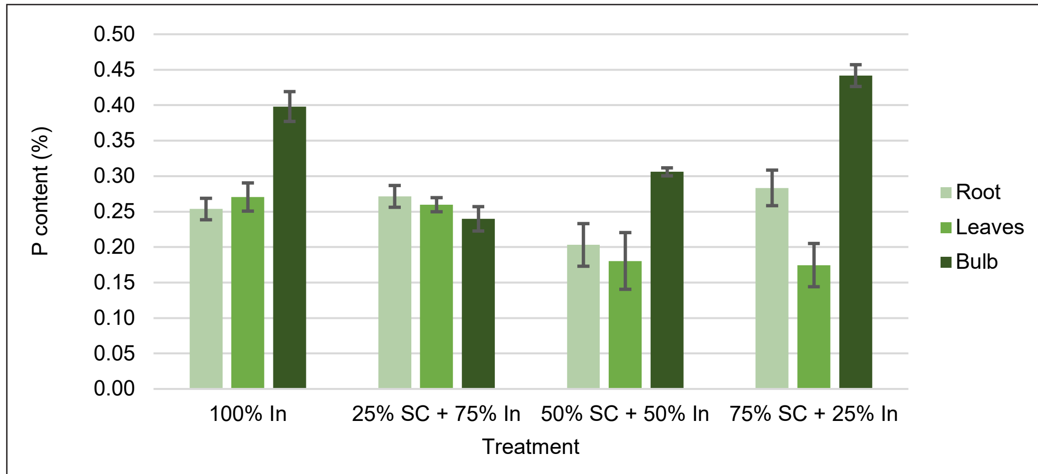


*Figure 2*. Tissue analysis of P content in roots, leaves, and bulbs with four treatments: 100% In = 100% inorganic fertilizer; 25% SC + 75% In = 25% *Sargassum* compost + 75% Inorganic fertilizer; 50% SC + 50% In = 50% *Sargassum* compost + 50% Inorganic fertilizer; and 75% SC + 25% In = 75% *Sargassum* compost + 25% Inorganic fertilizer, respectively

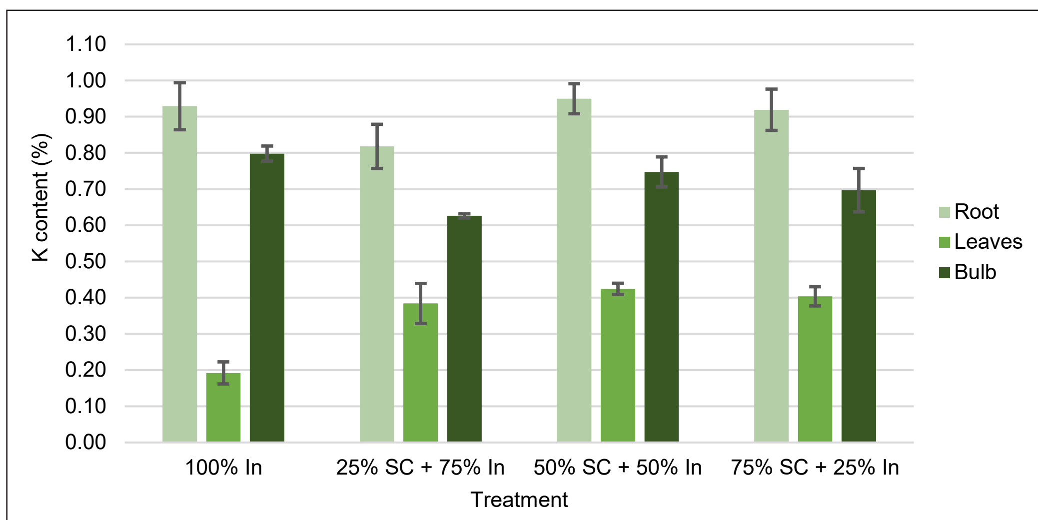


*Figure 3*. Tissue analysis of K content in roots, leaves, and bulbs with four treatments: 100% In = 100% inorganic fertilizer; 25% SC + 75% In = 25% *Sargassum* compost + 75% Inorganic fertilizer; 50% SC + 50% In = 50% *Sargassum* compost + 50% Inorganic fertilizer; and 75% SC + 25% In = 75% *Sargassum* compost + 25% Inorganic fertilizer, respectively

K in leaves plays a role in guard cells in stomata that regulate gas entry and exit (Hasanuzzaman et al., 2020). In addition, K plays a role in protein transporters and several enzymes involved in respiration and photosynthesis, so it affects plant growth (Ashley et al., 2006).

The highest Na accumulation was detected in the roots of all treatments (Figure 4). Elemental Na was observed because the

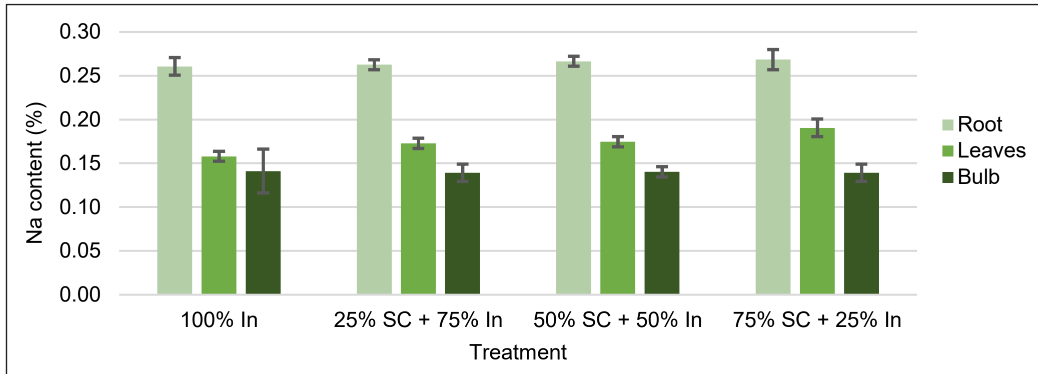


*Figure 4*. Tissue analysis of Na content in roots, leaves and bulbs with four treatments: 100% In = 100% inorganic fertilizer; 25% SC + 75% In = 25% *Sargassum* compost + 75% Inorganic fertilizer; 50% SC + 50% In = 50% *Sargassum* compost + 50% Inorganic fertilizer; and 75% SC + 25% In = 75% *Sargassum* compost + 25% Inorganic fertilizer, respectively

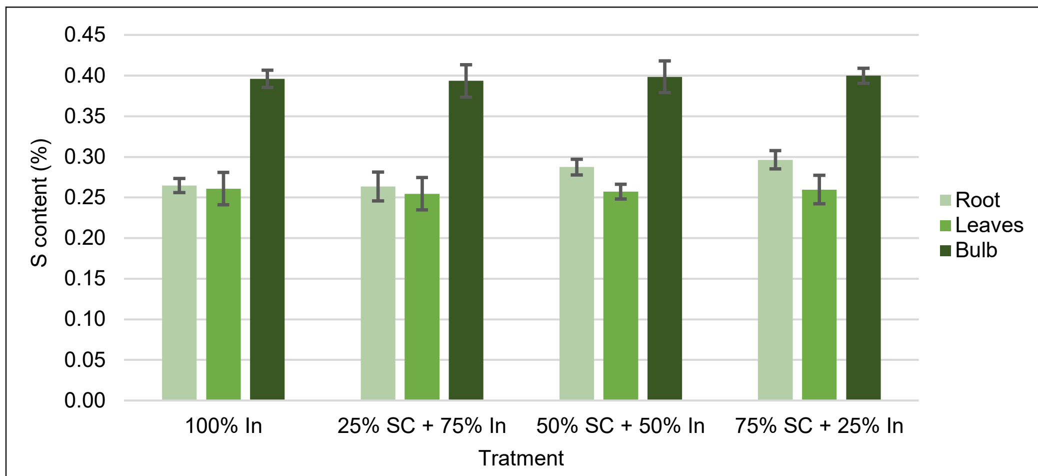


*Figure 5*. Tissue analysis of S content in roots, leaves, and bulbs with four treatments: 100% In = 100% inorganic fertilizer; 25% SC + 75% In = 25% *Sargassum* compost + 75% Inorganic fertilizer; 50% SC + 50% In = 50% *Sargassum* compost + 50% Inorganic fertilizer; and 75% SC + 25% In = 75% *Sargassum* compost + 25% Inorganic fertilizer, respectively

raw material for SC comes from marine organic material. Accumulation Na content in garlic roots could reach 0.03% (Astaneh et al., 2018). However, *Allium* species can absorb Na content depending on each genetic. The Na content has accumulated from the growing media, which contains Na, evidenced by the EC of the growing media (Table 3). Giving SC did not increase the Na in the growing media to an extreme. However, shallot of Bima Brebes variety accepted can adapt to high Na conditions as evidenced by Na content in root up to 27%. This result can relate to Syamsiyah et al. (2020) reported that the Bima Brebes variety shows better tolerance under salinity conditions or has a higher Na tolerance than other varieties. Furthermore, this result was

in accordance with Nabti et al. (2017), who reported that applying a large amount of seaweed fertilizer increases Na levels in plant tissues. Elemental Na is useful for increasing the osmotic potential, absorbing water, maintaining turgor, and stimulating plant growth, particularly under a potassium ion ($K^+$) deficiency (Pardo & Quintero, 2002; Wu, 2018). In addition, onion was grown under salinity conditions not given to affect the total S content (Aghajanzadeh et al., 2018). The highest S content (0.40%) was found in the bulbs of all treatments (Figure 5). S is a micronutrient that affects the quality of shallot bulbs for pungency related to the quantity of allicin. González-Morales et al. (2017) reported that S affects the sulfoxide in Allium plants which is an organoleptic quality for taste and sharpness.

## Growth and Yield

The growth parameters, as indicated by plant height, the number of leaves, and leaf area of shallot 56 days after planting in each treatment, are shown in Table 4. None of the shallot growth parameters was significantly different among the treatments. Because SC was able to substitute nutrients in the treatment of reduced doses of inorganic fertilizer 25% to 75%. SC contained 4.1% N, 0.5% P, 0.8% K, 7.2% Na, and 0.2% S (Table 2). The SC treatment provided the nutrients needed by the shallot plants. Therefore, SC reduced the inorganic fertilizer dose application. Reducing inorganic fertilizer use to 75% with the SC application resulted in the same growth as that of the treatment with 100% inorganic fertilizer. The good performance of shallot was also supported by the availability of hormones in the SC, including auxin, gibberellin, cytokinin, and kinetin (Table 1). These hormones stimulate growth through transcription factors or gene expression activities that affect morphological and physiological processes (Buttò et al., 2020).

The yield parameters, such as total fresh weight, total dry weight, and shallot bulb weight 65 days after planting, were not significantly different among the combined treatments (Table 5). The combination of organic and inorganic fertilizers prevents mineral loss so plants can absorb nutrients sufficiently and increase plant growth and yield (Liu et al., 2021). The combined fertilizer types were ideal for crop cultivation

Table 4
*Plant height, number of leaves, and leaf area of shallot 56 days after planting*

| Treatment | Plant height (cm) | Number of leave | Leaf area ($cm^2$) |
|---|---|---|---|
| 0% SC + 100% Inorganic | 39.98 ± 5.74a | 23.00 ± 3.50a | 362.45 ± 21.68a |
| 25% SC + 75% Inorganic | 47.43 ± 3.33a | 22.66 ± 8.28a | 339.41 ± 43.09a |
| 50% SC + 50% Inorganic | 45.61 ± 3.53a | 21.16 ± 6.64a | 316.46 ± 22.66a |
| 75% SC + 25% Inorganic | 42.45 ± 4.66a | 17.50 ± 8.26a | 309.66 ± 9.36a |
| CV (%) | 11.016 | 16.784 | 10.142 |

*Note*. Means followed by the same letters in the same column are not significantly different according to the LSD test; α = 5%

because inorganic fertilizers provide nutrients for plants during the early stages of growth, while the SC provided stable nutrients until the end of growth and filled the sinks of the crop organs. Therefore, our results show that shallot can be cultivated by reducing inorganic fertilizer to 75% of the recommended dose and replacing it with 25% SC.

Table 5
*Total fresh weight, total dry weight, and shallot bulb weight 65 days after planting*

| Treatment | Total of fresh weight (g) | Total of dry weight (g) | Bulbs fresh weight (g) |
|---|---|---|---|
| 0% SC + 100% Inorganic | 44.40 ± 12.08a | 4.22 ± 0.93a | 16.16 ± 5.33a |
| 25% SC + 75% Inorganic | 38.72 ± 8.07a | 4.41 ± 0.68a | 17.09 ± 4.32a |
| 50% SC + 50% Inorganic | 39.50 ± 9.21a | 4.23 ± 0.93a | 19.24 ± 4.57a |
| 75% SC + 25% Inorganic | 35.84 ± 9.51a | 3.85 ± 1.41a | 16.72 ± 7.37a |
| CV (%) | 13.294 | 15.005 | 11.547 |

*Note*. Means followed by the same letters in the same column were not significantly different according to the LSD test; α=5%

## CONCLUSION

SC contained of 4.1% N, 0.5% P, 0.8% K, 7.2% Na, 0.2% S, 8.14 $mg.g^{-1}$ auxin, 15.97 $mg.g^{-1}$ gibberellin, 7.70 $mg.g^{-1}$ cytokinin, and 2.78 $mg.g^{-1}$ kinetin. Substitution of 75% SC could increase 13% organic matter, 45% moisture content, and 87 $dS.m^{-1}$ EC of soil compared to 100% inorganic fertilizers, which gave the trend of increasing issue P, Na, and S content of shallot bulbs. Application of 25% to 75% SC substitution rate provided the same growth and yield of shallot as 100% inorganic fertilizer. Reducing 75% inorganic fertilizer use in shallot cultivation is possible by replacing 75% SC with organic fertilizer.

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# Endemic Muar, Malaysia Oyster *Crassostrea* (*Magallana*) *saidii* Wong & Sigwart, 2021 Approaches Optimal Harvest Despite Year-Round Multiple Recruitments

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## ABSTRACT

Population dynamics provides insight into how the population changes in structure over time. The insight is especially paramount for species with limited distribution and of conservation concern, such as *Crassostrea* (*Magallana*) *saidii* Wong & Sigwart, 2021 (Sigwart et al., 2021). The species is a recently described oyster endemic to the Muar estuary, Malaysia. The species' size-weight relationship and population structure were assessed in 2019 to determine its population dynamic. Results indicated that the oyster showed a negative allometric growth with a coefficient of 2.5422 ($b < 3$). The observed asymptotic height ($SH\infty$) was 15.23 cm, with a growth coefficient ($K$) of 0.69 per year. The low coefficient value was indicative of a slow growth rate, where it sets within the range of typically long-lived species. The oyster's growth performance index ($\varphi'$) was estimated at 2.204. The level of exploitation ($E$) index of the oyster (0.42) was lower than annual fishing mortality (1.27), indicating the *C.* (*M.*) *saidii* population was utilized close to the optimum yield. The oyster's recruitment pattern was continuous, peaking from April to June (18.46% to 13.82% oyster recruits) and September to November (10.68% to 12.37% recruits). The information of the current study is useful for sustainable management and proper utilization of the *C.* (*M.*) *saidii* oyster.

*Keywords*: *Crassostrea* (*Magallana*) *saidii*, mortality, optimal harvest, population dynamic, recruitment

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## INTRODUCTION

Oysters are ecologically a keystone species in the marine ecosystems by providing ecological services as reef-builders, filter feeders, and clarifiers, and for this first reason, they are known as bioengineers (Smaal et al., 2019). There are a few species of oysters found in Malaysia. The oyster *Crassostrea* (*Magallana*) *saidii* Wong & Sigwart, 2021 is a species new to science. It is morphologically and genetically distinct, limited to Sungai Muar, Malaysia, the only known population in the scientific record (Sigwart et al., 2021). Because of its creamy white meat and excellent quality, the species is known as the white oyster among fisherfolk and locals and is more popular with oyster fans than the *Crassostrea belcheri* (Axe Oyster) species, which can also be found in the estuary of Muar (Sigwart et al., 2021). The oyster fishery in Muar estuary was first reported in 1858 by a British Captain, who described Muar oyster as a delicacy reserved for the royals and high officials (Macpherson, 1858). It was sold at the Singapore market, about 200 km away (Hanitsch, 1908). However, later Muar oyster fishery was reported as “anemic” in 1929, probably due to the decline in the oyster population (Dover, 1929). Muar oysters have been farmed through bottom ranching by returning empty oyster shells to certain points of the Sungai Muar estuary, which is still practiced today. However, the small population is threatened by urbanization, pollution, and habitat destruction (Sigwart et al., 2021). Therefore, a sound biological baseline is required to ensure sustainability, protection, and management.

The length-weight relationship (LWR) is an intensive and efficient tool for identifying parameters in bivalve resources in the fishing industry (Peters, 1983). The LWR is useful for fishery biologists to predict biomass from length data and monitor population health (Cone, 1989; Pouladi et al., 2020). Parameters such as an oyster’s body weight and height to calculate the length-weight relationship (Osei et al., 2021). In addition, the stock assessment is effective for sound fisheries management. It is normally practiced to predict future yields and determine the exploitation levels of fish stocks based on certain fishing mortalities (King, 2007). After interpreting growth patterns and evaluating the natural stocks, the new species can be managed more effectively. The species’ economic management also depends on detailed and comprehensive information on population dynamics. However, the current study commenced to assess the size-weight relationship and population characteristics of C. (*M.*) *saidii* for the first-time utilizing Food and Agriculture Organization-International Center for Living Aquatic Resources Management stock assessment tools II (FiSAT II).

## METHODS

### Study Area

The river Sungai Muar flows through the states of Johor, Negeri Sembilan, and Pahang in Malaysia. The samples examined in this study were collected from the site

where shells of the oysters are traditionally used as cultch to encourage spat fall. The location of the sampling site was the Sungai Muar estuary, Johor, southern Malaysia (02°03'36.8"N, 102°34'18.7"E; Figure 1), one of the most important oyster producer areas in the country. This oyster generally settles on the riverbed, at curves of the river in strong currents, probably due to the lower siltation at high speed. The oyster is buried under sediment during unusual high sedimentation fluxes and can survive, keeping the ventral margin gape exposed to the water column (Sigwart et al., 2021).

**Samples Collection**

Samples were collected between January 2019 and December 2019 from the traditional fishers. They have dived into the water after anchoring their boat to 20 feet on wooden poles erected at designated oyster cultivating points in the estuary during low tides to collect oysters from the muddy bottom by handpicking.

**Oyster Measurement and Index**

The biometric measurements of the oysters were taken after the removal of algal biomass, encrusting organisms, and other waste materials at the ecology laboratory in the aquaculture department, Universiti Putra Malaysia (UPM). Three hundred seventy-one (371) specimens of *C.* (*M.*) *saidii* ranging in size from 5.87 cm to 14.12 cm and in weight from 36.8 g to 342.89 g were collected for the study. Shell height, the longest distance of the oyster, was measured using a vernier caliper, placing the jaw of the caliper to the hinge-to-bill length of the oyster shell (Galtsoff, 1964). In contrast, the total individual weight was weighed using a digital balance. The shell height of the oyster was used for the length-based analysis since the body weight of an oyster is more influenced by the shell height than the shell length and breadth (Nair & Nair, 1986; Osei et al., 2021).

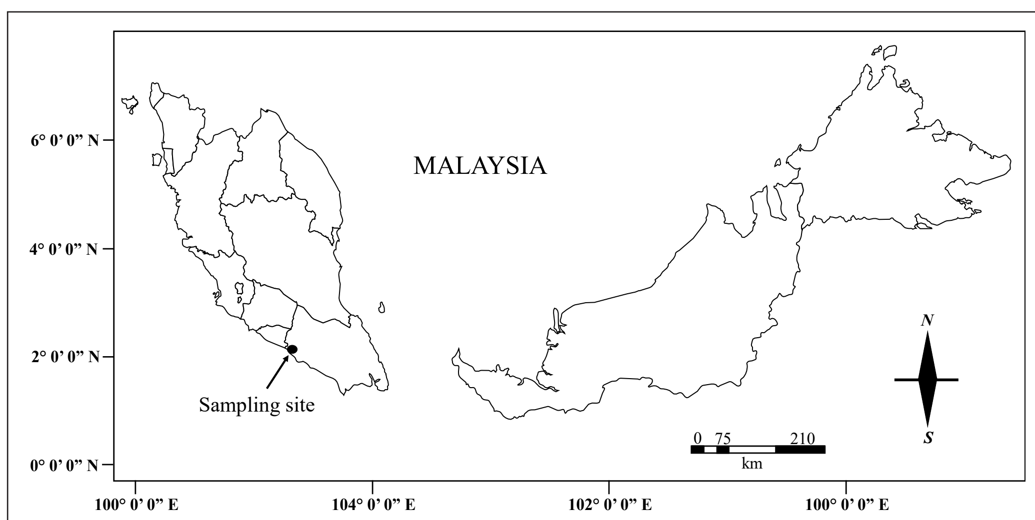


*Figure 1*. Location of the study site at Sungai Muar estuary, Johor, Malaysia (black circled)

**Statistical Analysis**

**Size-Weight Relationship.** The equation $TW = a(SH)^b$ (Quinn & Deriso, 1999; Ricker, 1975) was used to estimate the relationship between shell height (SH, cm) and total weight (TW, g), where *a* is the condition factor, and *b* is the relative growth rate. The least-squares linear regression on a log–log-transformed data, $Log_{10}\ TW = Log\ a + b(Log\ SH)$, was used to assess the parameters *a* and *b*. A *t*-test was applied to analyze the deviation of the relative growth rate of the Muar Oyster from the isometric value using the equation: $Ts = (b - bi) / SE$ (Kandeel et al., 2013), where *Ts* = value of the *t*-test, *b* = relative growth rate of *C.* (*M.*) *saidii*, *bi* = isometric value (3), and SE = standard error of the relative growth. The coefficient of regression ($R^2$) at 95% confidence limits was calculated as a determinative of the characteristics of the linear regression.

**Growth Parameters.** The collected data were assembled into height groups at intervals of 1 cm. The FiSAT II computer package was used to scrutinize the recorded height-frequency data, according to Gayanilo et al. (1996). Electronic Length Frequency Analysis-1 (ELEFAN-1) was applied to assess the asymptotic height ($SH\infty$) and growth coefficient (*K*) of the von Bertalanffy growth function (VBGF) (Pauly & David, 1981). A routine *K*-scan was used to determine the *K* value. Finally, the equation: $\varphi' = 2Log_{10}SH\infty + Log_{10}K$ was applied to estimate the growth performance index of *C.* (*M.*) *saidii*, where $\varphi'$ = growth performance index, $SH\infty$ = asymptotic height, and *K* = growth coefficient (Pauly & Munro, 1984).

The height-at-age curve was estimated by applying a non-linear least squares estimation procedure by fitting the VBGF using the equation: $SH_t = SH\infty\ [1 - e^{-k\ (t - to)}]$ (Pauly et al., 1992). In the equation, $SH_t$ = height at age *t*; $SH\infty$ = asymptotic height; *K* = growth coefficient; *t* = age of the oyster, and $t_0$ = the theoretical age when the oyster show null height (Newman, 2002). The hypothetical age at which the *C.* (*M.*) *saidii* reaches null height ($t_0$) was assessed using the equation: $Log\ (-t_0) = -0.3922 - 0.2752Log_{10}\ SH\infty - 1.038Log_{10}\ K$ (Pauly, 1979), where $SH\infty$ = asymptotic height and *K* = growth curvature of the VBGF curve.

**Mortality Pattern and Exploitation Rate.** The total mortality coefficient (*Z*) value of the oyster fishery was determined by applying the length-converted catch curve method (Pauly, 1987). The empirical equation assessed the annual natural mortality coefficient (*M*) of the oyster: $Log_{10}\ M = -0.0066 - 0.279Log_{10}\ SH\infty + 0.6543Log_{10}\ K + 0.4634Log_{10}\ T$ (Pauly, 1980); where *M* = annual natural mortality, $SH\infty$ = asymptotic height, *K* = VBGF growth coefficient, and *T* = annual environmental mean water temperature of the Muar estuary. The water temperature was recorded using an Honest Observer by Onset (HOBO) data logger, and the annual mean (± SE) was recorded at 30.1 ± 0.6 °C. The equation: $F = Z - M$ was used to estimate the fishing mortality of the oyster, where *F* = fishing mortality, *M* = natural mortality, and *Z* = total mortality

coefficient. The level of exploitation (*E*) was determined by the ratio of the fishing mortality to the total mortality, i.e., $E = F/Z = F/(F + M)$.

**Recruitment Pattern.** The recruitment pattern of the fishery was ascertained in FiSAT II software, inputting the growth parameters, namely growth coefficient (*K*), asymptotic length ($L\infty$), and theoretical age ($t_0$) at which the oyster reaches zero height. Then, the composite length-frequency distributions were decomposed by using a modified analytical method for mixtures of normal distributions (NORMally Separation [NORMSEP]) (Pauly & Caddy, 1985) in FiSAT II software.

## RESULTS

### Height-Weight Relationship

The height-weight relationship of the oyster was determined from the height and weight of the individuals, which was linear with high significant linearity (Figure 2; $R^2 = 0.9937$, $p < 0.01$). The slope of the relationship ($b = 2.5422$; SE = 0.012) is significantly different from 3 ($t = 38.93$, $p < 0.01$), which indicates a negative allometry growth of the *C.* (*M.*) *saidii* in the estuary. The linear regression revealed the average *b* value was 2.5422 ± 0.012 (SE), and the growth coefficient (*b*) varied between 2.5190 and 2.5653 at a 95% confidence level.

### Growth Parameters

The observed extreme height of the oyster was 14.50 cm, whereas the predicted extreme height of the oyster was 14.72 cm (Figure 3a). The range of extreme values was 13.73 cm to 15.72 cm at a 95% confidence interval. The Electronic Length Frequency Analysis 1 (ELEFAN 1) program reckoned growth coefficient (*K*) and asymptotic height ($SH\infty$) of VBGF for the oyster were 0.69 per year and 15.23 cm, respectively. The ELEFAN-1 analysis determined the hypothetical age

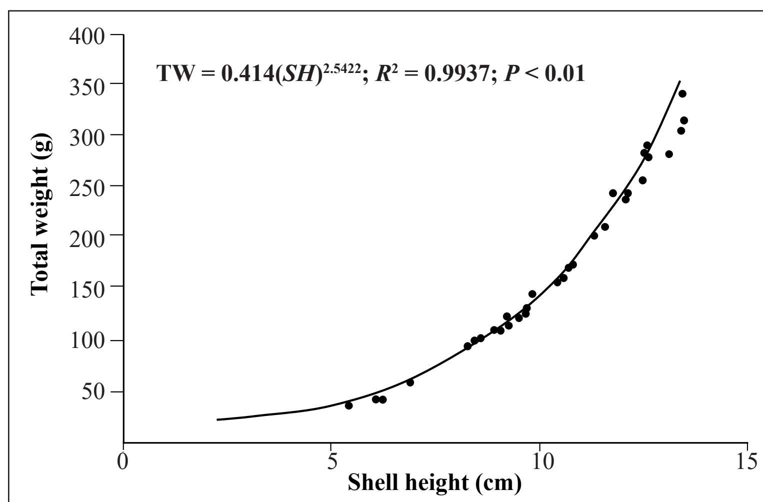


*Figure 2*. Height-weight relationship of *C.* (*M.*) *saidii* collected from the estuary of Sungai Muar, Johor, Malaysia

($t_o$) at which the oyster's null height was -0.28 years. However, the sizes attained by the oyster from Sungai Muar estuary, Johor were 4.68 cm, 6.35 cm, 8.94 cm, 12.07 cm, 13.65 cm, 14.43 cm, and 14.85 cm at the end of 0.25, 0.5, 1, 2, 3, 4, and 5 years of age, respectively (Figure 3b). The computed growth curve was superimposed with the growth parameters over the restricted height distribution (Figure 3c). The black bars in Figure 3c indicated higher frequencies, whereas the white bars indicated lower frequencies of the collected oyster during the study period in the Sungai Muar estuary. The oyster's growth performance index ($\varphi'$) was determined at 2.204 (Figure 3d).

## Mortality Pattern and Exploitation Rate

The total mortality rate (*Z*) was determined at 3.02 per year, while the natural mortality coefficient (*M*) and fishing mortality coefficient (*F*) were 1.75 and 1.27 per year, respectively (Figure 4). Therefore, the oyster's exploitation level (*E*) was obtained at 0.42, which appeared to be lower than

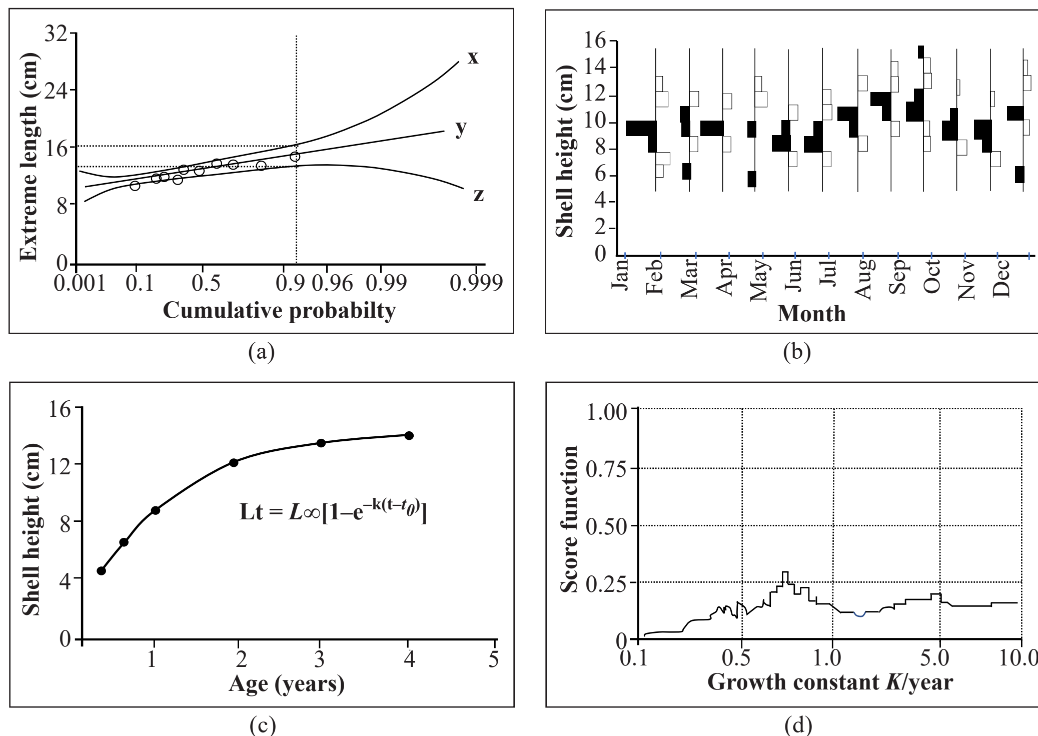


*Figure 3*. Growth parameters of the oyster *C.* (*M.*) *saidii*: (a) The predicted maximum height for the *C.* (*M.*) *saidii* according to the extreme value theory (Formacion et al., 1991). The overall asymptotic height was incised from the x and y, as well as y and z lines, respectively, to get the predicted asymptotic height and the extreme height at a 95% confidence level; (b) Plot of age and shell height relationship for the *C.* (*M.*) *saidii* in the estuary of Sungai Muar, Johor based on the von Bertalanffy growth function; (c) Restructured height-frequency distribution of *C.* (*M.*) *saidii* from the estuary of Sungai Muar Johor, Malaysia ($L\infty$ =15.23 cm and $K$ = 0.69 per year); (d) *K*-scan routine for the determination of the growth coefficients (*K*) and asymptotic height ($SH\infty$) of *C.* (*M.*) *saidii* using ELEFAN-1

the established expected minimum level of exploitation ($E$ = 0.50).

### Recruitment Pattern

The recruitment pattern of the oyster was continuous over the study period (Figure 5). At the beginning of the study in January, 5.47% oysters were recruited into the population. Two major recruitment pulses were observed from April to June (18.46% to 13.82%) and September to November (10.68% to 12.37%). The peak pulse of recruitment was observed in April, with 18.46% recruitment, whereas the minimum recruitment was produced in August, with 2.09% recruitment.

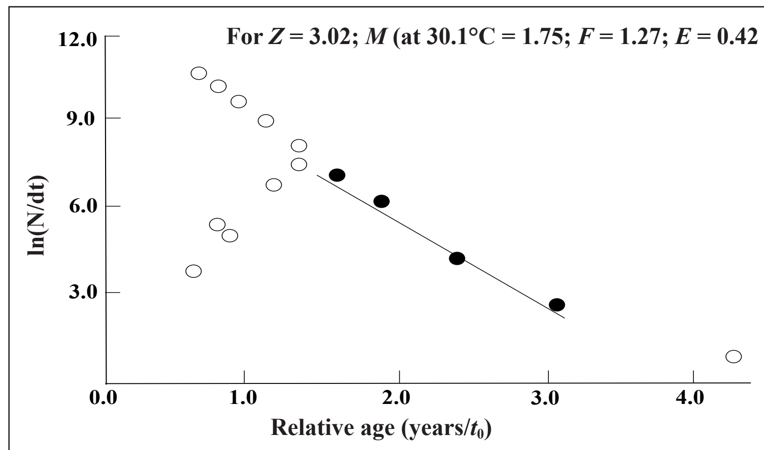


*Figure 4*. The size converted catch curve of the *C.* (*M.*) *saidii*, where 'N' is the population size and 'dt' is the time required to reach the population size. The least-square linear regression was applied to establish the catch curve. The solids dots points were used in the estimation, while the open dots reflect the relative ages that were either not fully recruited or close to asymptotic height ($SH\infty$)

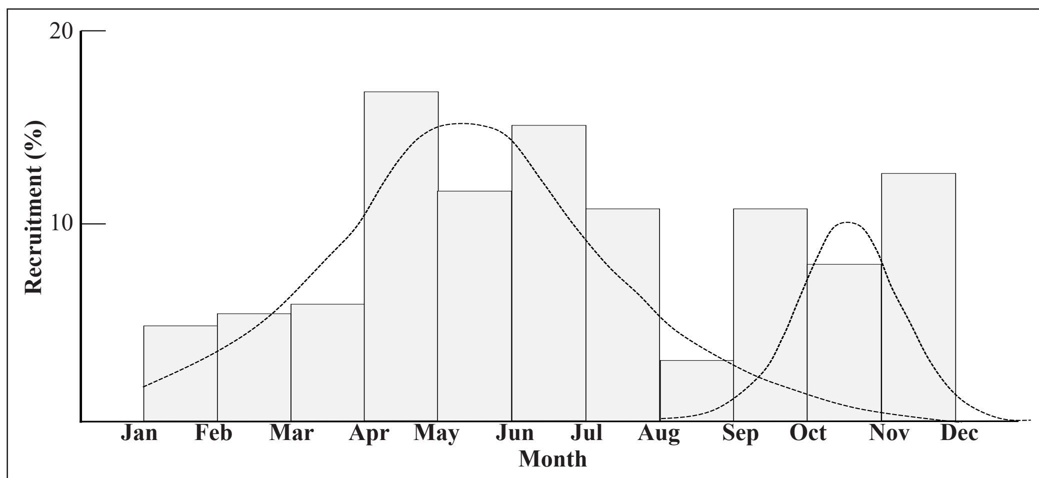


*Figure 5*. Bimodal distribution of recruitment pattern of *C.* (*M.*) *saidii* from the estuary of Sungai Muar, Johor Malaysia. The backward projection of the restricted height-frequency data was explored to get the pattern of the recruitment basis on a one-year timescale

## DISCUSSION

It is the first study on the size-weight relationship and population dynamics of the oyster *C.* (*M.*) *saidii.* Our study recorded the value of growth coefficients (*b*) for *C* (*M.*) *saidii* at 2.5422. Carlander (1977) reported that the value of *b* usually lies between 2.5 to 3.5 for aquatic animals. The growth of an aquatic animal is isometric when the relative growth rate (*b*) value is equal to 3 (Quinn & Deriso, 1999). This result implies that the weight gain of the oyster *C.* (*M.*) *saidii* was lower than the height increment in the Sungai Muar, Johor, Malaysia estuary. The growth of the oyster could be impacted by the influences of many anthropogenic activities, including effluent and industrial discharges, due to urbanization and pollution in the studied area.

In the current study, the asymptotic height was computed at 15.23 cm, and the growth coefficient *K* was calculated at 0.69 per year for the oyster *C.* (*M.*) *saidii*. These values did not differ much from some other *Crassostrea* species (Table 1). The asymptotic length ($L\infty$) of *Crassostrea madrasensis* was recorded at 20.88 cm in Bangladesh (Amin et al., 2008) and 14.90 cm for *Crassostrea rhizophorae* in Colombia (Mancera & Mendo, 1996). The annual growth rate (*K*) was recorded at 0.30 for the oyster *C. tulipa* (Osei et al., 2021) in Densu Delta, Ghana, and the value was observed at 3.96 for the oyster *C. rhizophorae* in the waters of Venezuela (Angell, 1986). These variations in the growth parameters can be explained by the different species or the environmental factors present in various study areas (Derbali et al., 2020).

The overall average first-year growth rate of the oyster was estimated at 0.48 (± 0.03) cm per month, and thus the oyster attained 8.94 cm in height in 12 months. The oyster's growth performance index ($\varphi^{/}$) was calculated at 2.204, suggesting that the oyster's culture could be feasible in the region due to its high growth performance.

A stock's yield is said to be optimized when the value of fishing and natural mortality are equal (Gulland, 1971). In the study, the natural mortality ($M$ = 1.75) was greater than the fishing mortality ($F$ = 1.27), suggesting an unequal position of the oyster stock as well as predation in the study area. Quayle (1980) noted that predators of oysters are crabs, snails, fish, flatworms, and starfish in the marine environment, while crabs may be the main predators in

Table 1
*Growth parameters of some* Crassostrea *species from different studies*

| Location | Species | $L\infty$ (cm) | $K$ yr-1 | $\varphi^{/}$ | Source |
|---|---|---|---|---|---|
| Bangladesh | *Crassostrea madrasensis* | 20.88 | 0.35 | 2.18 | Amin et al. (2008) |
| Venezuela | *Crassostrea rhizophorae* | 7.60 | 3.96 | 4.34 | Angell (1986) |
| Colombia | *Crassostrea rhizophorae* | 14.90 | 0.90 | 4.30 | Mancera and Mendo (1996) |
| Ghana | *Crassostrea tulipa* | 16.97 | 0.30 | - | Osei et al. (2021) |
| Malaysia | *Crassostrea* (*Magallana*) *saidii* | 15.23 | 0.69 | 2.204 | Present study |

*Note*. $L\infty$ = Asymptotic length; $K$ = Annual growth coefficient; $\varphi^{/}$ = Growth performance index

the estuary. The young oyster will survive if it grows rapidly to a size that exceeds the range where predation is effective. This number appeared to be small for the *C.* (*M.*) *saidii* in the Sungai Muar estuary due to high natural mortality. The higher natural mortality contrasted with fishing mortality might also be attributed to habitat degradation and habitat modification in the estuary for C. (*M.*) *saidii*. The exploitation rate (0.42) was close to the optimum level of exploitation (0.5) (Gulland, 1971), which indicates that the stock was harvested close to the optimum level.

The recruitment pattern obtained in the present study indicates that the continuous recruitment consisted of two seasonal peaks, the first between April to June and the second between September and November. This recruitment pattern is typical in tropical regions for the short-lived and fast-growing bivalved species (Mohammed & Yassien, 2003). In Malaysia, there is a lack of published reports on the recruitment pattern of *C.* (*M.*) *saidii*. Nevertheless, few studies have been conducted with different bivalved species in Malaysia to observe the recruitment pattern using the FiSAT II computer software package. For example, Al-Barwani et al. (2007) studied *Perna viridis* in Malacca, Malaysia found that the major recruitment was in July-August. The variations may be explained by Nair and Nair (1986), who reported that spawning and recruitment patterns could vary from species to species.

## CONCLUSION

The recruitment pattern of the *C.* (*M.*) *saidii* oyster was continuous and peaked twice throughout the year. The pattern predictably ensures year-round multiple recruitments in the Muar estuary. The natural mortality higher than the fishing mortality measures showed that predation affected the oyster population more than the harvest. Therefore, the dynamics of the *C.* (*M.*) *saidii* oyster population in the Muar estuary can currently sustain exploitation, such as from artisanal fishing practices.

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# Effects of Ultrasound and Steam Explosion Treatments on the Physicochemical Properties of Rice Bran Fibre

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## ABSTRACT

Rice bran (RB) is an underutilised fibre source due to undesirable effects when incorporated into food products. Thus, this study aims to improve the physicochemical properties of RB by using ultrasound (US) and steam explosion (SE) treatments, making it more usable in food applications. The US treatment of unpurified RB resulted in inconsistent average particle size, water binding capacity (WBC), and swelling capacity (SC). The bulk density (BD) decreased while the oil binding capacity (OBC) increased as the amplitude and time increased. While the purified rice bran resulted in decreased average particle size and BD; and increased WBC, SC, and OBC. The surface microstructure of the unpurified and purified rice bran became more porous, and the colour of the RB was darkened proportionally to the intensity of US treatment. The average particle size of unpurified increased while the purified RB increased after steam explosion treatment regardless of the intensity. The SE treatment also decreased WBC and SC of unpurified and purified RB, but no changes were observed on the surface microstructure of both samples. The BD of unpurified RB decreased, while the BD of purified RB increased after SE treatment. The SE treatment also resulted in a decrease in the OBC of purified RB, but no significant ($p > 0.05$) improvement was observed in the OBC of unpurified RB. Ultrasound brought these changes in the two treatments more effectively than steam explosion. The alteration of physicochemical properties of RB by the US and SE treatment in this study will allow it to be more applicable in the formulation of food products.

*Keywords*: Fibre, physicochemical, pretreatment, rice bran, steam explosion, ultrasound

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## INTRODUCTION

Rice bran is a by-product of the rice milling industry and is currently underutilised. Rice bran oil is the main product that has been commercialised, while some fat-free rice bran is utilised as stockfeed (Chinma et al., 2015; Ghosh, 2007). Currently, other uses of rice bran include nutritional supplements and ingredients of microbiological media (Hansawasdi & Kurdi, 2017; Sharif et al., 2014). Like other cereal bran, rice bran is rich in dietary fibre, as it contains up to 35% on a dry matter basis (Daou & Zhang, 2012). However, despite its high dietary fibre content, limited research has explored the functional properties of rice bran fibre, Even less research has attempted to improve its physicochemical and functional properties.

Studies showed that the incorporation of rice bran in the bread formulation led to a decrement in bread volume and had a detrimental effect on the texture and colour of the bread (Sharif et al., 2014). Physicochemical properties, such as bulk density, particle size, surface area, water binding capacity, swelling capacity, fat binding capacity, and solubility of rice bran fibre, play important roles in the processing and sensory properties of the products in which the fibre is incorporated. They can also significantly affect the health-related physiological functions of rice bran. Several studies have reported that chemical, physical, and enzymatic treatments can modify and improve the physicochemical properties of rice bran fibre (Daou & Zhang, 2011; Lebesi & Tzia, 2012; Qi et al., 2016; Rafe et al., 2017). Physical treatments offer many advantages over chemical treatments, such as lower costs, environmental friendliness, and industrially more practical (Brodeur et al., 2011). Among the physical treatments, ultrasound and steam explosion are particularly noteworthy for their ability to disrupt and breakdown the polymeric structure of cereal bran fibre, whereby increasing the fibre solubility by generating soluble oligosaccharides and enhancing the technological and health functionalities of the fibre (Daou & Zhang, 2012; Jiang & Guo, 2016).

However, the effects of ultrasound (US) and steam explosion (SE) treatments on the physicochemical properties of rice bran fibre have rarely been studied extensively. Furthermore, it has been reported that the purity of bran fibre may impact the effectiveness of some physical treatments due to the presence of starch and protein in the fibre in native bran (Qi et al., 2015), but these aspects have not been properly examined for US and SE treatments of rice bran. Ultrasound treatment is hypothesised to be able to breakdown the rice bran fibre caused by the acoustic cavitation where the microbubbles produced by ultrasound collided with the fibre, collapsed, and produced immense local energy, while the SE treatment is believed to degrade the lignocellulosic material of the rice bran due to the use of high temperature and pressure with a sudden release of the pressure. Therefore, both treatments are expected to alter the physicochemical properties of rice bran fibre.

Therefore, the objectives of this study were to assess the effect of US and SE treatments on the physicochemical properties of rice bran fibre with respect to the purity of the rice bran. In addition, this study also will provide evidence on how ultrasound and steam explosion treatments may improve the physicochemical properties of rice bran fibre for future use in food products.

## MATERIALS AND METHODS

### Rice Bran Samples, Chemicals, and Materials Used

Rice bran (RB) used in this study was obtained from SunRice (Australia). Upon collection from the milling and polishing process, the RB was stabilised by heat-treatment (drum dryer) and, after cooling to room temperature (25 ± 2 °C), packed in sealed polyethene bags with a brown paper outer cover. Following that, the bran samples were kept in containers packed with ice blocks for approximately 24 h during transportation to our laboratory at The University of New South Wales, Sydney, Australia. After arriving at the laboratory, the RB was vacuum-packed in 500 g polyethene bags and stored at -18 ºC before use. Ethanol and *n*-hexane were purchased from Ajax Chemical Pty. Ltd. (Australia). Corn oil was purchased from local supermarkets. Water used in all experiments was purified by reverse osmosis using the Mili-Q® reverse osmosis (RO) system (Australia) and henceforth referred to as Mili-Q® water.

### Defatting and Purification of Rice Bran

RB defatting was done following the procedure of Uraipong and Zhao (2016) with a minor modification. Firstly, the RB was dispersed in *n*-hexane (1:5, w/v), mixed in an incubator shaker at 250 rpm for 20 min, followed by centrifugation (Avanti J. E. Centrifuge Series, Beckman Coulter, USA) at 9,600 x *g* for 20 min and the *n*-hexane decanted. This procedure was repeated twice, and the defatted RB was first air-dried in a fume hood overnight to remove residual hexane by evaporation. Next, the bran samples were dried at 60 ºC overnight in an oven and finally stored in sealed polyethene bags at 4 °C before purification. Finally, purifying RB fibre was done by removing starch and protein following the procedure described by Hu et al. (2015) with minor modifications.

### Physical Pretreatment of Rice Bran

Physical treatment of RB involved two treatments, which were ultrasonic cavitation and steam explosion.

**Ultrasound Cavitation.** Ultrasound (US) cavitation treatment was applied to defatted and purified RB using a sonication immersion probe (20 kHz and 450 W, Branson Sonifier 450, USA). Defatted RB was dispersed in water at a ratio of 1:30 w/v in a 250 mL beaker, and the treatment was performed at three different power amplitudes, 60%, 80%, and 95%, and for 5, 10, 15, and 20 min for each power setting. The beaker containing the sample was partially immersed in an ice water bath

to ensure that the samples did not become overheated. The temperature of the slurry during the treatment was maintained at 25 ± 5 °C. The sonicated samples were freeze-dried (Leybold Lyovac GT2, Germany), sieved to pass 450 µm mesh size, and the samples were stored at -18 °C until further analysis. The freeze-dried RB was manually crushed with a spatula instead of grinding before sieving to prevent any additional mechanical effect on the RB fibre properties.

**Steam Explosion.** Instant catapult steam explosion treatment (SE) of the bran samples (defatted and purified RB) was performed using a QBS-80 batch SE apparatus (Hebi Steam Explosion Research Centre, China). Bran samples (50 g) were treated at 0.3 MPa (144 °C) and 0.6 MPa (165 °C) and for 120 s and 180 s. All treated samples were freeze-dried (Christ Alpha 1-2LD, Germany), sieved to pass 450 µm mesh size and kept at -18 °C for further analysis. The freeze-dried RB was manually crushed with a spatula instead of grinding before sieving to prevent any additional mechanical effect on the RB fibre properties.

### Analysis of Physicochemical Properties of Rice Bran

**Average Particle Size.** The average particle size of RB fibres was determined by using scanning electron microscopy (SEM) images equipped with Nano Measure 1.2 Software (Fudan University, China) adapted from W. Wang et al. (2019) with a slight modification. The software was set up for the unit of particle size required (µm), and the counting of the particle size (based on the SEM images) and the average particle size of the sample were calculated automatically by the software. The measurements were done in triplicate.

**Surface Microstructure.** Examination of the microstructure of RB fibres before and after the ultrasound cavitation and steam explosion treatments was done using SEM according to the procedure described by Wen et al. (2017) with slight modifications. First, samples of RB fibre were placed and spread into a thin layer on a specimen holder with the help of double-sided scotch tape and sputter-coated with gold (5 min, 30 mm thickness). Finally, each sample was transferred to a microscope (SEM, Hitachi S3400, Japan), where it was observed at an accelerating voltage of 20 kV.

**Bulk Density.** The bulk density (BD) of RB fibres was analysed by Chau et al. (2007). The BD was recorded as a ratio of the weight (g) of the RB sample to its volume (mL).

**Colour.** The colour of the RB fibre before and after treatment was measured with a Minolta CR-400 Chromameter with a Xenon lamp as the light source (Konica, Japan) adapted from Kurek et al. (2017) with a minor modification. The L*, a*, and b* colour values were recorded by the instrument, with the L value (0–100) representing lightness on the surface, while a* and b* values representing the chromatic components of redness to greenness and blueness to yellowness that range from -120 to 120, respectively.

**Water Binding Capacity.** The extracted fibre's water binding capacity (WBC) was determined by the method described by Robertson et al. (2000) using an external centrifugal force with minimum modification. WBC was expressed as the amount of water retained per gram dry sample.

WBC (g/g) = (Residue hydrated weight after centrifugation - Residue dry weight) / Residue dry weight

**Swelling Capacity.** The swelling capacity (SC) of RB was analysed using the method of Robertson et al. (2000). The swelling capacity (SC) was expressed as mL per g of dry sample.

SC = Volume occupied by sample (mL) / Weight of the original sample (g)

**Oil Binding Capacity.** Oil binding capacity (OBC) was measured using a method adapted from Abdul-Hamid and Luan (2000). The oil binding capacity was expressed as absorbed oil per gram sample.

**Statistical Analysis.** The experiments were repeated twice, and all data were collected in triplicate. Data were analysed by one-way analysis of variance (ANOVA) to determine the significant differences, and Tukey pairwise comparisons were used to compare the significant difference between the treatments. The statistical analysis was performed using Minitab version 17 (USA).

## RESULTS AND DISCUSSION

### Average Particle Size

It has been reported that the drying of bran fibre can influence its particle size due to particle agglomeration that occurs during the drying process (Beck et al., 2012). Therefore, to consider the drying effect, the untreated unpurified RB was dispersed in the same amount of deionised water as ultrasound-treated samples and freeze-dried (FD). This sample is referred to as untreated FD (Table 1). The average particle size of unpurified untreated RB FD was 83.3 ± 10.1, which was not significantly ($p > 0.05$) different from the unpurified RB without freeze-drying, which was 78.274 ± 11.0 µm. Thus, it is worth noting that the changes in particle size of RB in this study were not due to the freeze-drying process.

Unpurified RB showed an increase in average particle size after treatment at 60% US amplitude as treatment time increased (Table 1). Meanwhile, after 80% US amplitude treatment, the average particle size only increased after 10- and 15-min treatments and then reduced with no significant ($p > 0.05$) difference with the untreated RB after 20 min treatment. For treatment at 95% amplitude, the average particle size continued to increase as the treatment time increased.

Purified RB without treatment had an average particle size of 188.9 ± 21.67 µm (Table 2). It was significantly ($p < 0.05$) bigger than the unpurified RB and showed that the purification process caused swelling of the particle as it had been soaked in water,

Table 1

*The average particle size of unpurified RB after US treatment at different amplitudes and treatment times*

| US amplitude (%) | Treatment time (min) | Average particle size (μm) |
|---|---|---|
| 0 | 0 | [1]78.3 ± 11.0$^{A, c, y, Z}$ |
| 0 | 0 | [2]83.3 ± 10.1$^{A, bc, y, Z}$ |
| 60 | 5 | 101.1 ± 5.9$^{b}$ |
| | 10 | 105.0 ± 3.3$^{b}$ |
| | 15 | 134.5 ± 3.4$^{a}$ |
| | 20 | 128.4 ± 11.0$^{a}$ |
| 80 | 5 | 83.2 ± 3.0$^{y}$ |
| | 10 | 115.0 ± 6.8$^{x}$ |
| | 15 | 114.3 ± 8.0$^{x}$ |
| | 20 | 84.8 ± 3.1$^{y}$ |
| 95 | 5 | 176.8 ± 4.8$^{X}$ |
| | 10 | 184.5 ± 9.9$^{X}$ |
| | 15 | 150.6 ± 8.2$^{Y}$ |
| | 20 | 174.3 ± 15.7$^{X}$ |

*Note*.
[1]Untreated defatted RB without being freeze-dried
[2]Untreated FD = Defatted RB and freeze-dried
$^{A}$ = Means with different letters within the same column differ significantly ($p < 0.05$)
$^{a-c}$ = Means with different letters within the same column differ significantly ($p < 0.05$)
$^{x-y}$ = Means with different letters within the same column differ significantly ($p < 0.05$)
$^{X-Z}$ = Means with different letters within the same column differ significantly ($p < 0.05$)
Values of average particle size are means ± standard deviation (S.D.); $n = 3$

sodium hydroxide solution (NaOH) and undergone heat treatment during the process. After US treatment at all three amplitudes (60%, 80%, and 95%), the average particle size of purified RB decreased significantly ($p < 0.05$) compared to the untreated sample. However, no significant ($p < 0.05$) differences were observed between the 60% and 80% US amplitudes treatment times. At 95% US amplitude for treatment, the changes in average particle size fluctuated over the treatment time. However, all of them were still smaller than the untreated RB. This finding was in agreement with the previous studies by Hu et al. (2015) and Sumari et al. (2013). They observed similar effects of US treatment on the particle size of purified wheat bran and cellulose.

The average particle of unpurified RB after SE treatment was increased compared to untreated RB (Table 3). In contrast, the SE treatment decreased particle size for purified bran, similar to the effect of US treatment on purified bran. However, there were no significant ($p < 0.05$) differences between the particle sizes when the steam pressure was increased from 0.3 MPa to 0.6 MPa for both unpurified and purified RB.

Both US and SE are severe physical treatments that can significantly alter the particle size of RB. US produce a large number of microbubbles through acoustic cavitation, which, when collapsed, generate a high intensity of local energy (Hromádková et al., 2002; Sumari et al., 2013), thus degrading the particles of the material into smaller sizes. It was reflected in the reduction of particle size of the purified RB when treated by the US. However, the decrease in the particle size of the RB did not correlate with the US intensity. Furthermore, longer treatment times for the same US amplitude did not lead to a further reduction in the particle size. In contrast, higher US amplitude resulted in a smaller reduction in the particle size. These results appear to suggest a limit regarding

the effect of US treatment on the particle size of purified RB. If the treatment intensity was too high, agglomeration could occur. Meanwhile, the reduction in particle size after SE treatment of purified RB was due to the sudden release of the high pressure applied, which would shatter the bran particles into smaller pieces. To the best of our knowledge, no study has reported the effect of SE on the particle size of cereal bran. However, Yu et al. (2014) observed the reduction of particle size of taro pulp after high-pressure homogeniser treatment, which broadly agrees with our findings.

On the other hand, the increases in the particle size of unpurified RB by both US and SE treatments were most likely due to starch and protein, which swelled when dispersed in the water. In addition, the size of starch granules increased after high-pressure treatment due to starch gelatinisation (B. Wang et al., 2008; W. Wang et al., 2016).

Table 2

*The average particle size of purified RB after US treatment at different amplitudes and treatment times*

| US amplitude (%) | Treatment time (min) | Average particle size (µm) |
|---|---|---|
| 0 | 0 | 188.9 ± 21.7$^{A, a, x}$ |
| 60 | 5 | 88.1 ± 1.5$^{B}$ |
| | 10 | 98.3 ± 17.7$^{B}$ |
| | 15 | 80.0 ± 6.3$^{B}$ |
| | 20 | 87.2 ± 6.3$^{B}$ |
| 80 | 5 | 102.2 ± 15.9$^{b}$ |
| | 10 | 126.3 ± 18.2$^{b}$ |
| | 15 | 120.6 ± 14.9$^{b}$ |
| | 20 | 131.4 ± 17.7$^{b}$ |
| 95 | 5 | 112.1 ± 0.9$^{z}$ |
| | 10 | 153.9 ± 9.1$^{y}$ |
| | 15 | 119.9 ± 5.4$^{z}$ |
| | 20 | 148.9 ± 7.8$^{y}$ |

*Note.*
$^{A-B}$ = Means with different letters within the same column differ significantly ($p < 0.05$)
$^{a-c}$ = Means with different letters within the same column differ significantly ($p < 0.05$)
$^{x-z}$ = Means with different letters within the same column differ significantly ($p < 0.05$)
Values of average particle size are means ± standard deviation (S.D.); $n = 3$

Table 3

*The average particle size of unpurified and purified RB after SE treatment*

| Rice bran | Pressure (MPa) | Treatment time (min) | Average particle size (µm) |
|---|---|---|---|
| Unpurified | 0 | 0 | [1]78.3 ± 11.0$^{A,b}$ |
| | 0 | 0 | [2]83.3 ± 10.1$^{A,b}$ |
| | 0.3 | 2 | 216.8 ± 5.9$^{a}$ |
| | 0.6 | 2 | 221.7 ± 17.1$^{a}$ |
| Purified | 0 | 0 | 188.9 ± 21.7$^{a'}$ |
| | 0.3 | 2 | 120.8 ± 18.6$^{b'}$ |
| | 0.6 | 2 | 141.4 ± 29.8$^{a'b'}$ |

*Note.*
[1]Untreated defatted RB without freeze-dried
[2]Untreated FD = Defatted RB and freeze-dried
$^{A-B}$ = Means with different letters within the same column differ significantly ($p < 0.05$)
$^{a-b}$ = Means with different letters within the same column differ significantly ($p < 0.05$)
$^{a'-b'}$ = Means with different letters within the same column differ significantly ($p < 0.05$)
Values of average particle size are means ± standard deviation (S.D.); $n = 3$

**Changes in the Surface Microstructure**

The surface microstructure of unpurified and untreated RB (original) showed a packed, hard, and granular shape (Figure 1 A). Meanwhile, small shrinkages were observed on the untreated FD sample, but the porosity of the RB showed no changes (Figure 1 B). This finding is partly in agreement with the results reported by Y. Liu et al. (2017), who also observed shrinkage of the soluble fraction but, at the same time, found the formation of the porous structure on the insoluble fraction of orange peel fibre after freeze-drying. The difference was likely due to the different types of dietary fibre used in the two studies, and heat treatments were given to the fibre.

After the US treatment, the surface microstructure of unpurified RB became porous, broken into smaller pieces, flaky, and flat-shaped, and this effect became more apparent when the US amplitude and time were increased from 60% to 95% and 5 to 20 min, respectively (Figure 1 C - N). A similar disruption in the structure of chestnut polysaccharides after US treatment was observed in previous studies (Hou et al., 2016; Ying et al., 2011).

In contrast, SE treatment did not give an impact as significant as the US on the surface structure of RB. There were no changes in the surface structure of RB when treated at 0.3 MPa for 2 min. However, a small change in porosity was observed when treated at the higher pressure of 0.6 MPa for the same length of time, as shown in Figure 1 O-P. This result is in disagreement with the finding observed in wheat bran (Jiang & Guo, 2016). However, the parent reason for the different effects observed was not clear and could be due to the different types of fibre used in their studies, which had different cell wall strengths. Furthermore, Jiang and Guo (2016) applied higher pressures (up to 3.7 MPa) in their study, which was much higher than the pressure used in this study. Therefore, the relatively low steam pressure used in this study might not be enough to cause significant changes to the surface structure of RB.

It is found that some shrinkage and small holes occurred in the structure of untreated RB after the purification (Figure 2 A). It could be due to the soaking and high temperature applied during the purification process. After the US treatment, the same effect as the unpurified RB was observed, where the porosity increased as the US amplitude and time increased (Figure 2 B - M). Moreover, similar to the effect observed with unpurified bran, the purified bran treated by SE did not show noticeable changes in the microstructure (Figure 2 N - O). It again can be related to the lower pressures used in this study compared to the previous study by Jiang and Guo (2016), who applied higher pressure on wheat bran (1.0 - 3.7 MPa).

**Bulk Density**

For unpurified RB, with treatments at 60% and 80% US amplitudes, at all US times, the BD decreased by about 50%–60% (data not shown) from the untreated RB. However, no significant ($p > 0.05$) difference between these two amplitudes

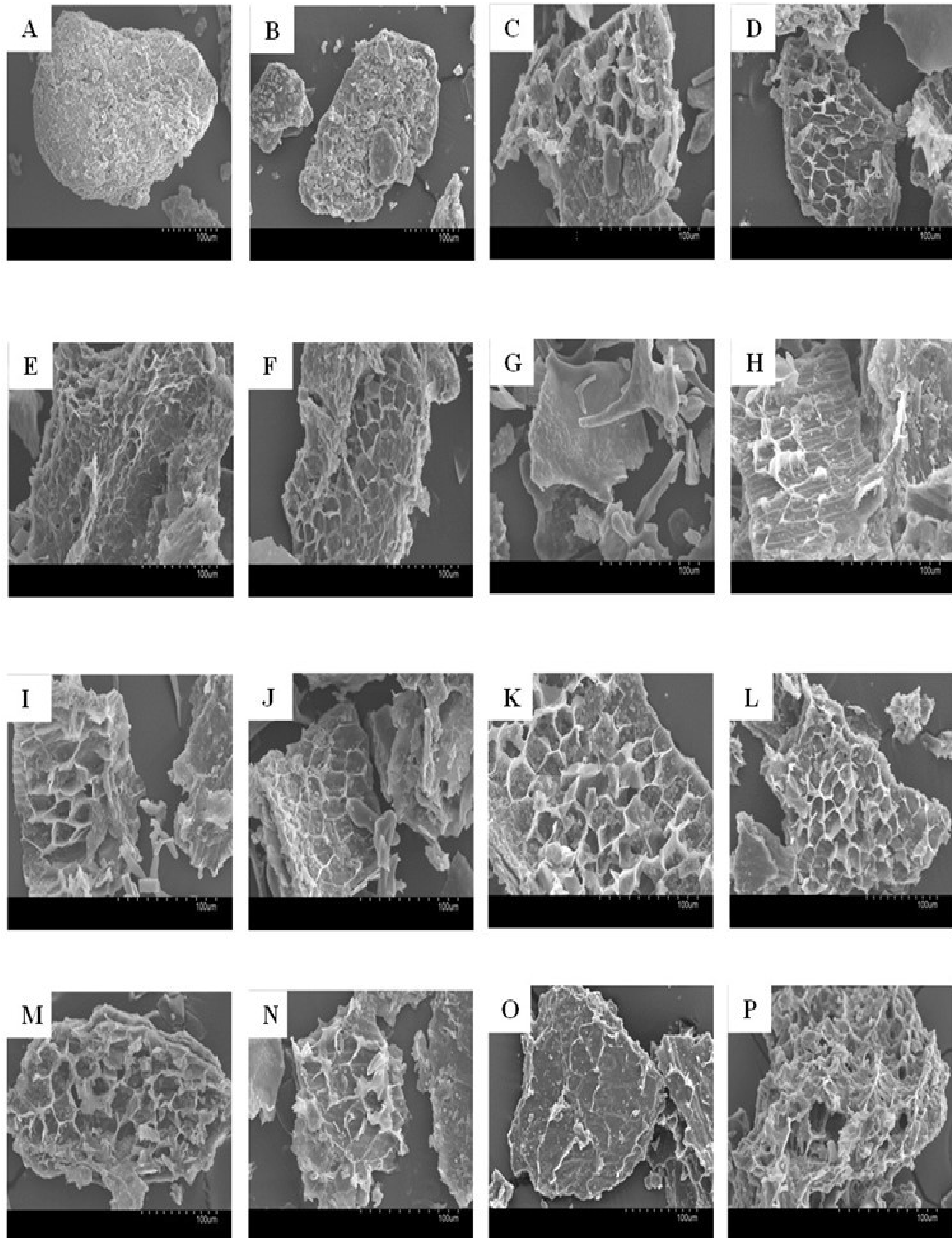


*Figure 1*. SEM images of un-purified RB fibre after ultrasound and steam explosion treatment

*Note*. A = Untreated; B = Untreated freeze-dried (untreated FD); C = Ultrasound at 60% amplitude for 5 min (same for the following); D = 60%, 10 min; E = 60%, 15 min; F = 60%, 20 min; G = 80%, 5 min; H = 80%, 10 min; I = 80%, 15 min; J = 80%, 20 min; K = 95%, 5 min; L = 95%, 10 min; M = 95%, 15 min; N = 95%, 20 min; O = Steam explosion at 0.3MPa, 2 min; P = Steam explosion at 0.6MPa, 2 min

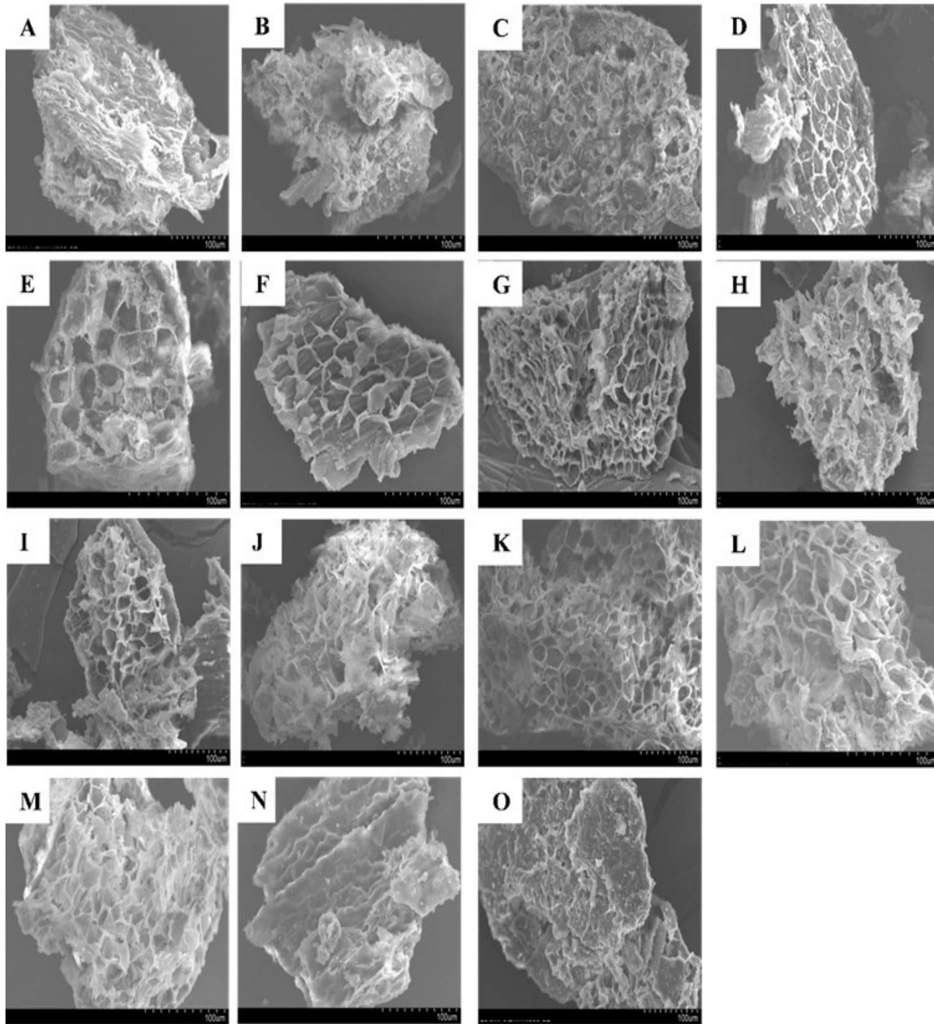


*Figure 2*. SEM images of purified RB fibre after ultrasound and steam explosion treatment

*Note*. A = Untreated; B = Ultrasound treated 60% amplitude for 5 min (same for the following); C = 60%, 10 min; D = 60%, 15 min; E = 60%, 20 min; F = 80%, 5 min; G = 80%, 10 min; H = 80%, 15 min; I = 80%, 20 min; J = 95%, 5 min; K = 95%, 10 min; L = 95%, 15 min; M = 95%, 20min; N = 0.3MPa, 2min; O = 0.6MPa 2 min

was observed. However, when the US amplitude was increased to the maximum (95%), the reduction of the BD was less than at the previous two amplitudes, at around 42%–48% (data not shown). Nevertheless, no significant differences ($p > 0.05$) in the BD at all US amplitude were observed when US treatment time was increased from 5 to 20 min.

After purification, the BD of untreated RB was reduced by about 45% compared with the unpurified bran, from 0.53 g/mL to 0.23 g/mL. The bulk density of purified RB steeply decreased with increment of US

amplitude and time of treatment (Figure 3B). However, no significant ($p > 0.05$) differences were observed between 60% and 80% amplitudes at all treatment times. The lowest BD was recorded (0.15 g/mL) with treatment at 95% for 20 min.

SE treated unpurified RB also showed a decrement of BD, but no significant ($p > 0.05$) difference was observed when the pressure was increased from 0.3 MPa to 0.6 MPa. The SE treatment only reduced the BD of unpurified RB by 20.5% compared to untreated RB (Figure 3C). In contrast, treatment of purified RB with the SE at 0.3 MPa and 0.6 MPa caused an increase in the BD by 17.9% (at both pressures) compared to the untreated RB.

Bulk density is a physical property that can be influenced by many factors, such as the porosity and size of particles as well as the processing methods used (Z. Liu et al., 2016). The lower bulk density of the purified RB compared to the unpurified counterpart indicated that the purification process had altered the structure of the bran by making it more porous, which is reflected in the SEM results (Figure 2A). It is expected as the removal of starch and protein from the bran would leave some space in the bran structure. Therefore, it is generally expected that bran with a more porous structure would have a lower BD. Moreover, a reduction in particle size would also lead to lower BD, as shown by many studies (Chau et al., 2007; Huang et al., 2010; T. Wang et al., 2012; Wang, Raddatz, et al., 2013). In this study, the porosity of RB decreased with the intensity of US treatment (as shown in section "Changes in the Surface Microstructure"). At the same time, its effect on the reduction in particle size was inconsistent and did not show a clear pattern. However, in the case of SE, the treatment caused an increase in particle size. Nevertheless, all treatments caused a reduction in the bulk density of the RB. In the case of US treatment, the reduction was more pronounced with increasing treatment intensity. These results demonstrated that the influence of porosity on the BD of RB fibre was greater than the particle size.

Moreover, BD has reportedly been influenced by drying methods such as oven drying or freeze-drying (Y. Liu et al., 2017). Therefore, the bulk density of freeze-dried untreated unpurified RB (untreated FD) was analysed to remove the possibility that the reduction in BD is an effect of drying. The BD of untreated FD was 0.374 g/mL (not shown in the graph), which was 29% ($p < 0.05$) lower than untreated RB that had not been freeze-dried. The bulk density of US-treated RB at all US amplitudes was significantly ($p < 0.05$) lower than the untreated FD. At amplitudes 60%, 80%, and 95%, the reduction of the bulk density was 39-45%, 30-41%, and 17-27% compared to untreated FD, respectively (data not shown). According to Y. Liu et al. (2017), freeze-drying led to the formation of a porous structure in dietary fibre, which would cause a decrease in its BD. However, in this study, the freeze-drying process did not significantly alter the porosity of untreated RB (Figure 1 B). Therefore, it indicates that the reduction in the bulk US treated RB

was not due to the freeze-drying process applied after the treatment; indeed, it was attributable to the increase in porosity and reduction in the particle size of the RB as a result of the treatment.

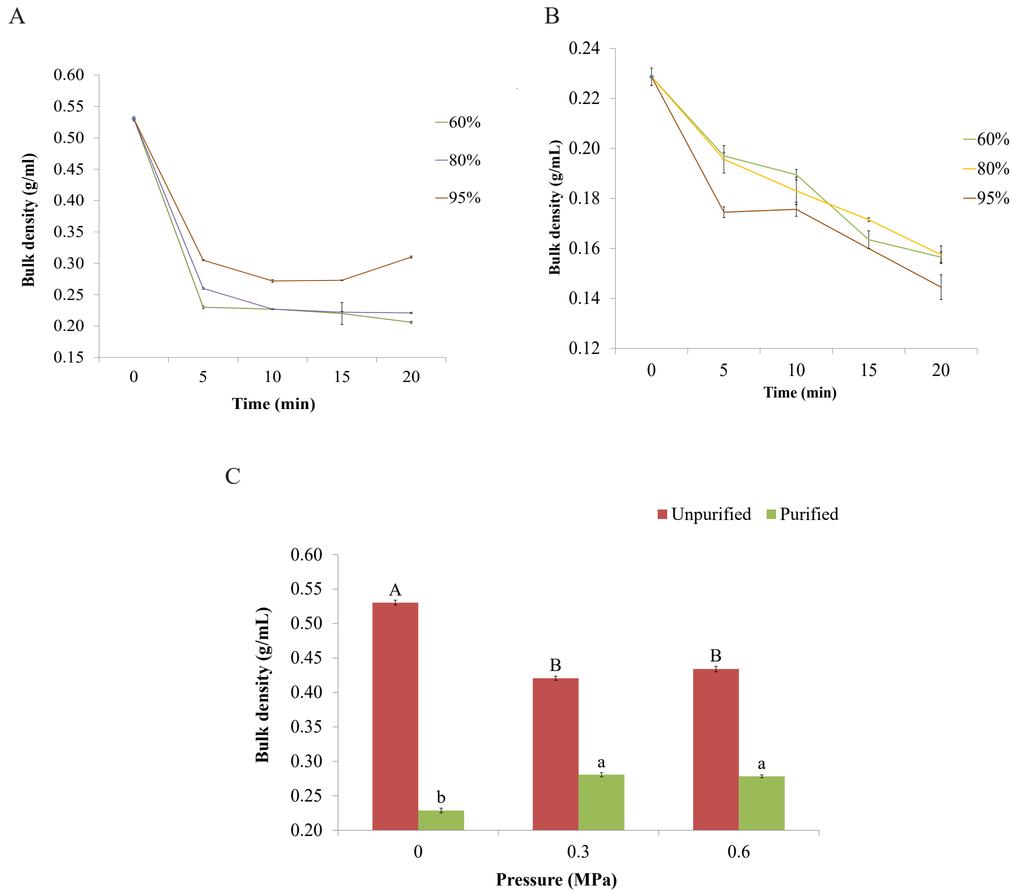


*Figure 3*. Bulk density

A) Bulk density of unpurified RB after ultrasound treatment
B) Bulk density of purified RB after ultrasound treatment
C) Bulk density of unpurified and purified rice bran after steam explosion treatment

*Note*.
A-B = Means with different letters are differ significantly ($p < 0.05$)
a-b = Means with different letters are differ significantly ($p < 0.05$)
Values are means ± standard deviation (S.D.). $n = 3$

## Colour Changes

A decrease in lightness (L* value) of unpurified RB was observed after US treatment at all amplitudes applied, and the decrease became greater as the US amplitude increased (Table 4). Furthermore,

the darkest colour of the bran was observed with the highest US amplitude (95%) and the longest time of treatment (20 min). Meanwhile, US treatment caused the a* value of the bran to increase, but the b* value did not change significantly ($p > 0.05$) after the treatment.

Purified RB had a lower L* value than the unpurified bran, likely attributable to the high temperature used during the gelatinisation steps to remove starch. After US treatment, a further decrease in L* values occurred. However, the L* values did not decrease as low as that observed in unpurified RB. Besides, the a* value also decreased as the US amplitude and time increased, with the lowest value observed at 95% amplitude and 20 min. In contrast, the b* value showed an increase compared to the untreated RB.

The colour of unpurified RB became darker after SE treatment compared to untreated and US-treated RB, which is evident by the lower L* values (Table 5). Furthermore, the L, a*, and b* values decreased as the steam pressure increased from 0.3 MPa and 0.6 MPa.

The colour values changes (L*, a*, b*) in RB after the US and SE treatment were mainly due to the Maillard reaction and sugars caramelisation generated by the starch and polysaccharides breakdown in the RB (Rosell, 2011). Although US is a nonthermal treatment, small rises in temperature of the bran were observed during the treatment, which became more pronounced with time, which would expect to accelerate the browning reaction. Severe browning was expected with the SE treatment due to the high temperatures involved, which was reflected in the large increases of the a* (redness) and b* (yellowness) values. When the purification process removed the starch and protein, which are key participants or precursors of Maillard browning and caramelisation, the magnitudes of darkening changes were much less, which were reflected in the lesser decreases of L* (lightness) values for purified RB.

Table 4

*Colour values of RB after US treatment*

| | US amplitude (%) | Treatment time (min) | Colour value | | |
|---|---|---|---|---|---|
| | | | L* | a* | b* |
| | 0 | 0 | $70.2 \pm 0.2^{A, a, v}$ | $3.6 \pm 0.1^{C, b, v}$ | $17.5 \pm 0.2^{A, b, w}$ |
| Unpurified bran | 60 | 5 | $66.4 \pm 1.0^{B}$ | $4.0 \pm 0.1^{A}$ | $17.7 \pm 0.3^{A}$ |
| | | 10 | $66.4 \pm 0.6^{B}$ | $4.1 \pm 0.1^{A}$ | $17.5 \pm 0.3^{A}$ |
| | | 15 | $66.6 \pm 0.2^{B}$ | $3.6 \pm 0.1^{C}$ | $17.9 \pm 0.2^{A}$ |
| | | 20 | $67.3 \pm 0.0^{B}$ | $3.8 \pm 0.1^{B}$ | $17.8 \pm 0.0^{A}$ |

Table 4 *(Continue)*

| | US amplitude (%) | Treatment time (min) | Colour value | | |
|---|---|---|---|---|---|
| | | | L* | a* | b* |
| | 80 | 5 | 67.1 ± 0.1$^{c}$ | 4.3 ± 0.1$^{a}$ | 18.4 ± 0.1$^{a}$ |
| | | 10 | 67.1 ± 0.4$^{c}$ | 3.8 ± 0.1$^{a}$ | 17.6 ± 0.1$^{b}$ |
| | | 15 | 68.4 ± 1.4$^{b}$ | 3.6 ± 0.0$^{b}$ | 17.3 ± 0.1$^{b}$ |
| | | 20 | 69.6 ± 1.3$^{a}$ | 3.6 ± 0.1$^{b}$ | 17.7 ± 0.2$^{b}$ |
| | 95 | 5 | 67.9 ± 0.1$^{w}$ | 3.8 ± 0.0$^{w}$ | 17.8 ± 0.0$^{vw}$ |
| | | 10 | 67.4 ± 0.1$^{x}$ | 3.7 ± 0.0$^{w}$ | 17.7 ± 0.0$^{vw}$ |
| | | 15 | 65.4 ± 0.5$^{y}$ | 3.9 ± 0.0$^{w}$ | 17.9 ± 0.0$^{v}$ |
| | | 20 | 65.1 ± 0.1$^{z}$ | 3.9 ± 0.0$^{w}$ | 17.5 ± 0.0$^{vw}$ |
| Purified bran | 0 | 0 | 68.4 ± 0.2$^{A, a, x}$ | 4.2 ± 0.0$^{A, a, v}$ | 19.2 ± 0.0$^{D, d, v}$ |
| | 60 | 5 | 68.3 ± 0.1$^{B}$ | 3.4 ± 0.1$^{B}$ | 21.3 ± 0.0$^{B}$ |
| | | 10 | 68.7 ± 0.7$^{B}$ | 3.4 ± 0.1$^{B}$ | 21.7 ± 0.1$^{A}$ |
| | | 15 | 69.4 ± 0.1$^{B}$ | 3.5 ± 0.0$^{B}$ | 21.8 ± 0.1$^{A}$ |
| | | 20 | 70.6 ± 0.7$^{B}$ | 3.2 ± 0.1$^{C}$ | 20.7 ± 0.1$^{C}$ |
| | 80 | 5 | 66.9 ± 0.1$^{b}$ | 3.5 ± 0.0$^{b}$ | 20.1 ± 0.0$^{c}$ |
| | | 10 | 66.9 ± 0.0$^{b}$ | 3.4 ± 0.2$^{c}$ | 20.2 ± 0.0$^{c}$ |
| | | 15 | 66.6 ± 0.2$^{b}$ | 3.4 ± 0.0$^{c}$ | 20.8 ± 0.0$^{a}$ |
| | | 20 | 65.5 ± 0.1$^{c}$ | 3.3 ± 0.1$^{c}$ | 20.6 ± 0.1$^{b}$ |
| | 95 | 5 | 67.3 ± 0.0$^{x}$ | 3.3 ± 0.1$^{w}$ | 20.6 ± 0.2$^{w}$ |
| | | 10 | 67.2 ± 0.1$^{x}$ | 3.3 ± 0.0$^{w}$ | 20.8 ± 0.2$^{w}$ |
| | | 15 | 67.7 ± 0.5$^{x}$ | 3.3 ± 0.1$^{w}$ | 21.4 ± 0.3$^{w}$ |
| | | 20 | 67.7 ± 0.1$^{x}$ | 3.0 ± 0.0$^{x}$ | 20.5 ± 0.2$^{w}$ |

*Note*.
Values within the same column with different letters were significantly different ($p < 0.05$) Values are means ± standard deviation (S.D.). $n = 3$
The means values were compared with the untreated (0 amplitude and 0 times) as a control for unpurified and purified, respectively
$^{A\text{-}D}$ = Means with different letters within the same column and the same US amplitude differ significantly ($p < 0.05$)
$^{a\text{-}d}$ = Means with different letters within the same column and the same US amplitude differ significantly ($p < 0.05$)
$^{v\text{-}z}$ = Means with different letters within the same column and the same US amplitude differ significantly ($p < 0.05$)

Table 5
*Colour values of RB after SE treatment*

| | Pressure (MPa) | Treatment time (min) | Colour value | | |
|---|---|---|---|---|---|
| | | | L | a* | b* |
| Unpurified bran | 0.0 | 0 | 70.2 ± 0.2[A] | 3.6 ± 0.1[C] | 17.5 ± 0.2[A] |
| | 0.3 | 2 | 55.8 ± 0.1[B] | 5.9 ± 0.1[B] | 16.3 ± 0.3[B] |
| | 0.6 | 2 | 51.4 ± 0.1[C] | 6.6 ± 0.1[A] | 16.3 ± 0.1[B] |
| Purified bran | 0.0 | 0 | 68.4 ± 0.2[a] | 4.2 ± 0.0[c] | 19.2 ± 0.0[b] |
| | 0.3 | 2 | 65.6 ± 0.6[b] | 4.6 ± 0.2[b] | 19.3 ± 0.4[b] |
| | 0.6 | 2 | 62.6 ± 0.5[c] | 5.1 ± 0.2[a] | 20.2 ± 0.9[a] |

*Note*.
Values within the same column with different letters were significantly different ($p < 0.05$)
The means values were compared with the untreated (0 amplitude and 0 times) as a control for unpurified and purified, respectively
[A-C] = Means with different letters within the same column differ significantly ($p < 0.05$)
[a-c] = Means with different letters within the same column differ significantly ($p < 0.05$)
Values are means ± standard deviation (S.D.). $n = 3$

**Water Binding Capacity**

Water Binding Capacity (WBC) of unpurified RB did not show a clear trend over the amplitude and time of US treatment (Figure 4A). The WBC of unpurified and purified RB before physical treatment was 3.1 and 6.3 g/g, respectively, showing that the purification process increased the WBC of RB by two folds. The purified RB showed a clear trend in the relationship between water binding capacity and the severity of US treatment, where an increase in treatment amplitude and time generally led to an increase in the water-binding capacity of the bran samples (Figure 4B). At 60% US amplitude, there was no significant ($p > 0.05$) effect on the WBC of purified RB fibres after 5, 10, and 15 min of treatment, but the WBC increased steeply to 7.9 g/g after 20 min of treatment and gave the highest WBC value. At the maximum amplitude (95%), the WBC ($p < 0.05$) increased significantly from 6.4 to 6.90 g/g after 5- and 10-min treatment, respectively, but no further significant increment was observed when the US time was increased to 15 and 20 min. It was also observed that there was no significant ($p < 0.05$) difference between WBC at 80% and 95% US amplitudes for 10 and 15 min of treatment.

In contrast, the WBC of both unpurified and purified RB decreased after SE treatment (Figure 4C). The WBC of unpurified RB before treatment was 3.1 g/g, reduced to 2.2 g/g and 2.4 g/g after being treated with the SE at 0.3 MPa and 0.6 MPa for 2 min,

respectively. For purified RB, the WBC was reduced to 3.3 g/g and 3.5 g/g. No significant ($p > 0.05$) difference was observed between the WBC of RB treated by the SE at the two different pressure levels.

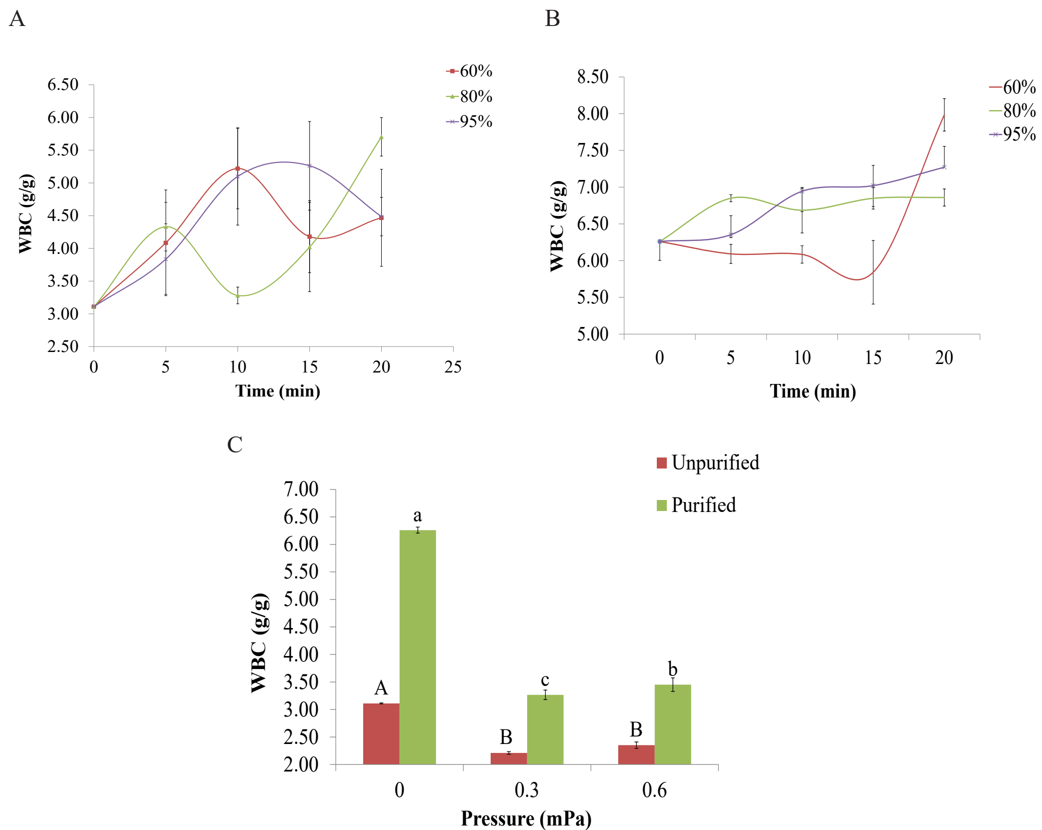


*Figure 4*. Water binding capacity
A) Water binding capacity of unpurified rice bran after ultrasound treatment
B) Water binding capacity of purified rice bran after ultrasound treatment
C) Water binding capacity of unpurified and purified rice bran after steam explosion treatment

*Note*.
A-B = Means with different letters differ significantly ($p < 0.05$)
a-b = Means with different letters differ significantly ($p < 0.05$)
Values are means ± standard deviation (S.D.). $n = 3$

## Swelling Capacity

Swelling capacity (SC) showed significant ($p < 0.05$) changes after the US treatment. At each amplitude, the SC of the RB increased with treatment time, reached a plateau, and then declined (Figure 5A). Surprisingly, US at 60% amplitude for 5 min resulted in the highest SC value for the unpurified

bran, which declined with further increased treatment time. The SC of bran treated at 80% and 95% amplitudes reached a peak at 20 and 5 min, respectively, and then declined.

Purification of RB caused its swelling capacity to increase by about 22% before physical treatment (Figure 5B). It is likely because the removal of starch and protein left more binding sites for water uptake (Qi et al., 2015, 2016). Furthermore, purification also made the RB more responsive to US treatment. At 60% US amplitude, the SC increased to 8.7, 9.2, and 9.2 mL/g after 5, 10, and 15 min of treatment, respectively. However, after 20 min of US treatment, the SC increased to the highest value compared to US treatment at 80% and 95% amplitudes. This trend was in agreement with the WBC results, where the highest WBC value was also observed at 60% US amplitude and 20 min, as described in the proceeding section. However, slightly different trends were observed at 80% and 95% amplitudes. At 80% US amplitude, the SC increased after 5 min of treatment, remained largely constant between 5 and 15 min, and increased again after that. Meanwhile, at 95% US amplitude, the SC increased as the time increased. Overall, the swelling capacity of purified RB was significantly ($p < 0.05$) higher after the US treatment.

For SE treatment, the SC of unpurified and purified RB decreased after the treatment, as shown in Figure 5C, similar to the results for WBC as described in the previous section. For the unpurified bran sample, the SC decreased from 5.7 g/g (untreated) to 4.9 g/g and 4.7 g/g after being treated at 0.3 MPa and 0.6 MPa, respectively. For the purified RB, the SC decreased from 7.3 g/g to 6.4 g/g and 6.5 g/g after treatment at the same pressure. However, the SC values did not differ significantly ($p > 0.05$) between treatments at the two pressures for both unpurified and purified RB.

### Oil Binding Capacity

The oil binding capacity (OBC) of untreated unpurified RB was 1.8 g/g. After 5 min of US treatment with different US amplitudes, the OBC significantly increased. With US applied at 60% and 80% amplitudes, the OBC showed no further significant changes ($p > 0.05$) when the treatment time was increased. However, with 95% US amplitude, the OBC increased continuously with increasing treatment time (Figure 6A).

The OBC of untreated RB increased to 4.1 g/g after purification (Figure 6B). Regarding the effect of US treatment on the OBC of purified RB, a different trend was observed from that of unpurified bran. After 5 min of US treatment at 60% and 95% amplitudes, no significant differences ($p > 0.05$) were observed in OBC compared to the untreated sample. At 60% amplitude, significant increases in OBC only occurred with a further increase in treatment time after 5 min, and the OBC increased sharply as the time increased from 5 to 20 min. At 95% amplitude, the maximum OBC was observed after 10 min of treatment and no further changes occurred as the treatment time increased. At 80% amplitude, the OBC

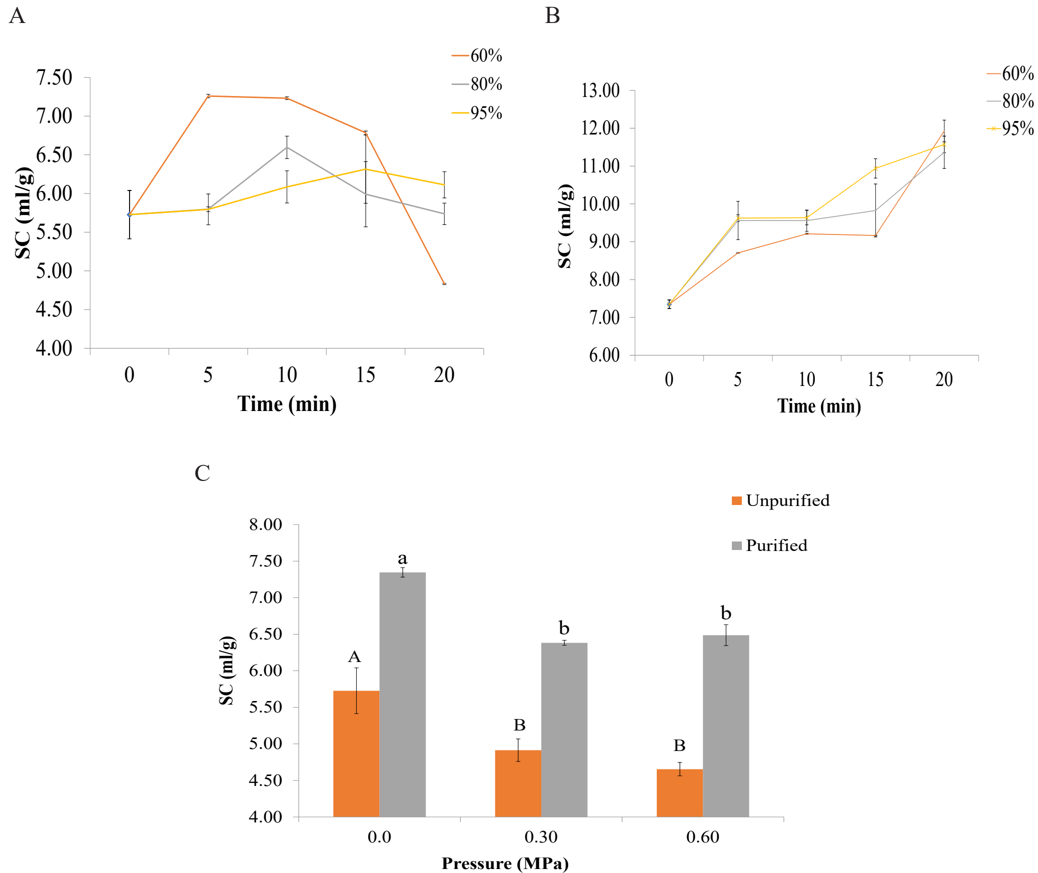


*Figure 5*. Swelling capacity
A) Swelling capacity of unpurified rice bran after ultrasound treatment
B) Swelling capacity of purified rice bran after ultrasound treatment
C) Swelling capacity of unpurified and purified rice bran after steam explosion

*Note*.
A-B = Means with different letters differ significantly ($p < 0.05$)
a-b = Means with different letters differ significantly ($p < 0.05$)
Values are means ± standard deviation (S.D.). $n = 3$

increased sharply after 5 min of treatment and no significant changes ($p > 0.05$) were observed with further increase in treatment time. The highest OBC was observed at 60% US amplitude and 20 min of treatment.

The OBC of SE-treated RB is shown in Figure 6C. Treatment with SE resulted in a significant decrease in the OBC of purified RB, and the reduction was greater at 0.6 MPa than at 0.3 MPa. However, for unpurified RB, the SE treatment showed no significant ($p > 0.05$) effect on OBC.

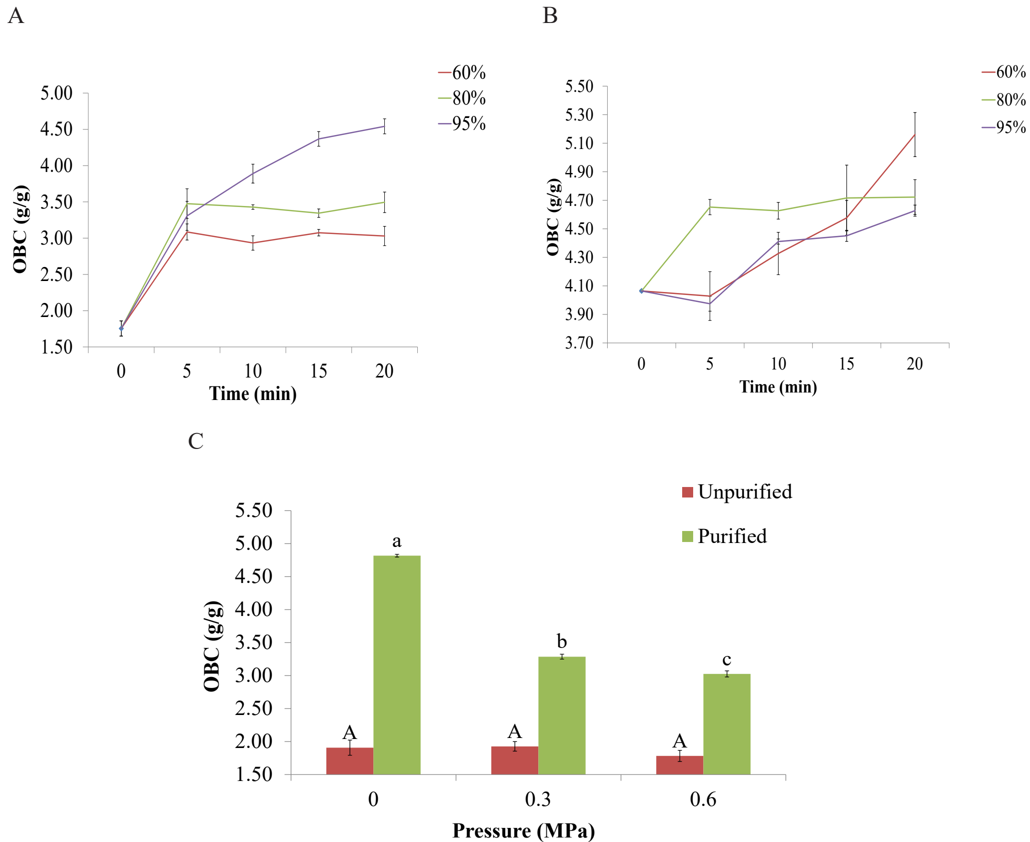


*Figure 6*. Oil binding capacity
A) Oil binding capacity of unpurified rice bran after ultrasound treatment
B) Oil binding capacity of purified rice bran after ultrasound treatment
C) Oil binding capacity of unpurified and purified rice bran after steam explosion treatment

*Note*.
A-B = Means with different letters differ significantly ($p < 0.05$)
a-b = Means with different letters differ significantly ($p < 0.05$)
Values are means ± standard deviation (S.D.). $n = 3$

## General Discussion on the Changes in the Physicochemical Properties of RB After US and SE Treatment

Water-binding capacity (WBC) and swelling capacity (SC) are important hydration properties of fibres as they perform important functions in food products as well as in human physiology (Blackwood et al., 2000; Chater et al., 2015; Rosell, 2011; Rosell & Santos, 2010; Sabanis et al., 2009). The ability of dietary fibre to retain water is strongly influenced by the chemical and structural properties of the fibre (Chaplin, 2003). The inconsistent trend of water binding capacity observed in unpurified RB after US treatment was likely due to the presence of starch and protein. Purifying RB by removing starch and protein significantly

increased the water-binding capacity even before physical treatment was applied. It is expected as the removal of impurities, such as starch and protein from dietary fibre would increase the porosity (as shown in Figure 2A), surface area and expose more hydroxyl and carboxyl groups and capillary spaces between the cell wall structures of dietary fibre, which would increase the water-binding capability (Qi et al., 2015, 2016).

The effect of US treatment on the WBC and SC of purified RB was rather complex and not straightforward. At lower US amplitudes, longer treatment times were needed to produce a significant effect on WBC and SC, but it could achieve a greater increase in WBC and SC with prolonged treatment. On the other hand, high US amplitudes could increase the WBC for a shorter treatment time, but the prolonged treatment did not make further gains in WBC. These results were likely attributable to the effect of US treatment on the physical and chemical properties of RB. Initially, an increase in US treatment intensity (amplitude and time) would cause an increase in the porosity of the fibre matrix, with consequent increases in WBC and SC. However, as the US intensity was increased further, the porosity of the fibre matrix might not increase further as the structure had been opened to its limit (as shown in Figure 5), and all possible binding sites were occupied by water. Our results agreed with the findings of Ulbrich and Flöter (2014), who observed insignificant increments in cellulose-based oat fibre product WBC, even long cycles of high-pressure homogenisation treatment were applied as the maximum level of porosity was achieved. High-intensity US treatment could also result in cleavage of glycosidic bonds in the main polymeric chain as well as in the branching units, with consequent loss of sugars from the fibre (Ebringerová & Hromádková, 2010). The breakdown of the main polymer chain and side chains means a loss of hydrogen bonding sites, which could reduce the fibre's ability to retain water, which explains the lower WBC of RB when treated at higher US amplitude and longer treatment times.

WBC and SC are also related to the particle size of the fibre. In separate studies, Stephen and Cummings (1979) and Wang, Sun, et al. (2013) reported that the reduction in the particle size of wheat and corn bran by grinding leads to increases in WBC and SC as more surface area became available for water binding and absorption. It is in broad agreement with our findings where the WBC and SC of purified RB treated by US increased, corresponding to a reduction of the bran particle size (Table 2). However, a contradicting effect was reported on ground ash gourd and radish fibres, where the decrease in fibre particle size resulted in increased WBC (Gupta & Premavalli, 2010). The difference might be due to the different chemical compositions of the different fibres studied (Gupta & Premavalli, 2010; Raghavendra et al., 2006). The WBC of purified and US-treated RB in this study was lower than the WBC of RB that was treated with sulphuric acid (10–22.45 g/g)

(Qi et al., 2015). It suggests that methods of fibre preparation can also impact the WBC of RB. However, for unpurified and US-treated RB, it is difficult to relate the particle size to the WBC and SC as they fluctuate due to the interference of starch and protein, as discussed already. The reduction in WBC and SC of RB after SE treatment was likely linked to the morphological changes, where the surface structure of SE treated RB became packed, shrunk, and less porous compared to untreated and US treated RB, as shown in Figure 1 O - P and Figure 2 N - O.

In summary, US treatment resulted in increases in the WBC of RB, and it could be valuable for application in food products to prevent syneresis, modify viscosity, texture, and mouthfeel characteristics, as well as reduce calories of formulated food products (Chau et al., 2006). Besides, the WBC of fibre has been demonstrated to play an important role in bakery products as it influences major events that occur during baking, such as starch gelatinisation, protein denaturation, gluten dilution, and formation of flavour and colour (Rosell, 2011; Rosell et al., 2010). In addition, water binding also retards moisture loss during the storage of baked goods, which helps slow down the staling process of the products, especially bread (Ranasalva & Visvanathan, 2014; Sabanis et al., 2009; Walter, 2014). In terms of physiological properties, the ability of dietary fibre to entrap water is closely related to the digestion process, such as gastric emptying, faecal bulk, and gut transit time (Chater et al., 2015; Davidson & McDonald, 1998; Takahashi et al., 2009). A greater SC is also a desirable physicochemical property to be included as a food ingredient as it can induce satiety and improve bowel movement (Kuan & Liong, 2008). The WBC of dietary fibre also may affect nutrient absorption, postprandial satiety, and intestinal motility (Jenkins et al., 1978).

Similar to hydration properties, oil binding capacity is also influenced by several factors, including surface structure, particle size, overall charge density, and hydrophobicity of the components. Wang, Sun, et al. (2013) concluded that increased porosity led to more surface exposure and enhancement of the physical entrapment of oil by capillary attraction. Our results generally support it (Figure 1 C - N, Figure 2 B - M). Besides the porosity, the increase in OBC of purified and US-treated RB could also be attributed to the reduction in the average particle size. These findings are in agreement with the report by Chen et al. (2013), who studied the effect of particle size reduction on the OBC of oat bran and peach and found that the OBC was improved after US treatment. Although the OBC of US-treated RB in this study was lower than the OBC of chemically treated RB reported by Qi et al. (2015), the improvement by the treatment was nevertheless significant and had practical value. According to a previous study, rice bran fibre with high oil binding capacity are desirable as it has the potential for use in products such as gluten-free bread, pasta, and meat products to prevent agglomeration, alter the food matrix and stabilise high-fat foods and emulsions

(Chinma et al., 2015; Elleuch et al., 2011; Kaur et al., 2012; Sairam et al., 2011; Saunders, 1985). In terms of physiological properties, OBC is believed to absorb oil and fat in the intestinal tract, as well as retain and remove the fat through faeces (Mora et al., 2013). This property is valuable in food product formulations that require good oil retention and cholesterol absorption (T. Wang et al., 2012).

Meanwhile, the SE treatment did not cause major changes to the porosity of RB (Figure 1 O - P; Figure 2 N - O) but increases in the particle size, consequently leading to a decrease in OBC. Our results are in disagreement with the findings reported by Shen et al. (2019), who studied the effect of SE treatment on the extraction and OBC of soluble fibres from black soybean hull. However, their study is not directly comparable with ours as they investigated the oil binding capacity of the soluble fibre fractions, while our study investigated the binding capacity of the total fibre.

## CONCLUSION

The US and SE treatments had a significant impact on the physical properties of RB, including particle size, surface microstructure, and BD. The treatments also led to significant changes in the physicochemical properties of the fibre, including WBC, SC, OBC, and colour. The changes in the physicochemical properties of the RB fibre were strongly affected by both the purity of the RB and treatment intensity. This study showed that the purified RB gave clear trends in the relationship between the US and SE treatments received and changes in the physicochemical properties. Purification also allowed the US and SE treatments to work more effectively on RB fibre to bring improvements in its physicochemical properties. Ultrasound brought these changes in the two treatments more effectively than steam explosion. With the knowledge gained from this study, further exploration of the modification of the physicochemical properties of RB by both treatments to vary the application of RB in food products that can enhance the health-promoting properties in the future.

## CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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# Effects of Salinity Sources on Growth, Physiological Process, Yield, and Fruit Quality of Grafted Rock Melon (*Cucumis melo* L.)

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## ABSTRACT

There is an increase in demand for high-quality rock melon for the local market. Supplementing salt with a nutrient solution is a viable approach that can be implemented to improve fruit quality. Therefore, this study aims to determine the best salt treatment that can be utilized to increase fruit quality without reducing growth, yield, and physiological process. The study is conducted by grafting (DAG) rock melon/bottle gourd at 18 days with four sources of salinity: basic nutrient solution (BNS) (2.5 dS $m^{-1}$), sodium chloride (NaCl) (50 mM) + BNS (7.1 dS $m^{-1}$), potassium nitrate ($KNO_3$) (50 mM) + BNS (7.1 dS $m^{-1}$), and high strength nutrient solution (NS) (7.1 dS $m^{-1}$). The plants were arranged in a randomized complete block design (RCBD) with four replications. Salinity induced using $KNO_3$ + BNS sustained most growth variables, fruit quality, relative water content, and leaf gas exchange compared with control. However, applying NaCl + BNS and high strength NS could sustain all physiological processes and increase fruit quality components, such as total soluble solid and sugar-acid ratio compared to control. Fruit weight had reduced regardless of salinity sources than those grown in control with their respective fruit weight reduction of 28.8%, 28.26%, and 27.72%. To conclude, incorporating NaCl at 50 mM

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is the most feasible approach to be applied on grafted rock melon/bottle gourd even though the fruit weight had reduced. It is due to the high fruit quality measured, capable of sustaining all physiological processes, provides lower cost, and is easily accessible than other sources of salinity.

*Keywords*: Fruit quality, grafted rock melon, salinity sources, salinity stress, salt-tolerant rootstock

## INTRODUCTION

Rock melon, also known as muskmelon (*Cucumis melo* L.), is a short-term horticultural crop and belongs to the Cucurbitaceae family. It is one of the most important crops commonly cultivated for whole fruit consumption. Generally, melons are used as vegetables, desserts, salads, and pickles (Pitrat, 2016). The fruits are highly nutritious and rich in antioxidants, including phenolic compounds, ascorbic acid, and carotenoids (Menon & Rao, 2012). It also contains vitamin C, flavonoids, vitamin B, and fiber and is naturally low in fat and sodium. In addition, it has no cholesterol and provides many essential nutrients, especially potassium (Manchali et al., 2021). Furthermore, melons used as vegetables have flavonoids, alkaloids, and bitter elements, which increase health advantages (Gómez-García et al., 2020).

In Malaysia, rock melon is commercially grown to fulfill a demand for the local and export markets. Over the years, rock melon production has increased drastically, up to 45.56% since 2012, with total production recorded at 5,845.71 metric tonnes in 2018 (Department of Agriculture Malaysia and Agro-based Industry Malaysia [DOA], 2018). The increase in production areas is due to high consumers' demand for high fruit quality. According to Lester (2006), fruit quality, such as sweetness, taste, texture, and flavor, is the most important reason for consumers' higher preference for melon productions.

Salt addition as a nutrient solution has high potential, is cost-effective, and is easy to be adapted, which can increase rock melon quality. The success has been proven for a variety of horticultural crop species including cucumber (Huang et al., 2009), tomatoes (Azarmi et al., 2010), and watermelon (Costa et al., 2013). Accumulation of salt may reduce water absorption capacity that could increase dry-matter components. An increase in dry matter components ultimately enhances fruit quality attributes, including total soluble solid, titratable acidity, and sugar-acid ratio (Dias et al., 2018). According to Jawandha et al. (2017), applying $KNO_3$ salts could increase vegetative growth and enhance yield and fruit quality attributes. Besides, high NaCl salt accumulated in the fertilization system has proven to increase fruit quality parameters, such as total soluble solid, total titratable acidity, and sugar-acid ratio (de L. Pereira et al., 2017). On the other hand, the nutrient solution is an inorganic fertilizer that delivers nutrients in the form of a liquid. It comprises many ionic or salt compositions important for sustaining plant performances in horticultural crop productions (Saliqehdar et al., 2014).

Nevertheless, rock melon's excess and continuous supply could lead to salinity development and deleteriously affect the growth and yield. Under a high saline environment, crop growth and yield are reduced significantly, impairing the physiological process (Munns & Tester, 2008). Pessarakli (2016) stated that the rock melon is moderately sensitive to salt stress among cucurbit species. Supplementation using different salinity sources can improve fruit quality without reducing growth, physiological process, and yield (P. Zhang et al., 2016). The salt-tolerant level in rock melon could be increased using salt-tolerant rootstock (Yarsi et al., 2017). Previously, bottle gourd has been proven to be the promising salt-tolerant rootstock for watermelon (Yetisir & Uygur, 2010), cucumber (Huang et al., 2009), and higher growth performance was recorded under salt stress than self-grafted plants. Thus, applying salinity sources on grafted rock melon/bottle gourd could be justified to improve fruit quality without detrimentally affecting growth, physiological process, and yield.

After considering those factors, the studies are necessary to identify and select the most suitable salinity sources for grafted rock melon/bottle gourd. This research may contribute to a new knowledge of growing rock melon with salt added, improving fruit quality without reducing growth, physiological process, and yield. Furthermore, the knowledge produced in this study may be useful in improving rock melon development practices and exploiting new research pathways for rock melon in the future.

## MATERIALS AND METHODS

### Location and Experimental Materials

This experiment was conducted in the rain shelter structure at University's Agriculture Park nursery, Universiti Putra Malaysia, from September 2020 to December 2020. The planting materials used in this study were rock melon (*Cucumis melo* L.) var. Glamour as scion and bottle gourd (*Lagenaria siceraria*) var. BG696 as rootstock.

### Treatments and Experimental Design

This experiment consisted of four salinity source treatments arranged in a randomized complete block design (RCBD) with four replications. The replications used were represented as a block to reduce the errors and interferences in the rain shelter structure. Each of the replicas consisted of eight plants, totaling 128 plants. The salinity sources treatments used in this study were basic nutrient solution (BNS) as control, NaCl, $KNO_3$, and high strength nutrient solution (NS), with their respective concentrations shown in Table 1.

The formulation for BNS is based on a standard nutrient solution used in the melon fertigation system, prepared at 2.5 dS $m^{-1}$ (Shahid et al., 2009). This solution contains (mg $L^{-1}$) of 232 nitrogen (N), 67 phosphorus (P), 239 potassium (K), 120 calcium (Ca), 30 magnesium (Mg), 3 iron (Fe), 80 sulfur (S), 0.62 manganese (Mn), 0.44 boron (B), 0.02 copper (Cu), 0.11 zinc

Table 1
*The salinity sources treatments with respective concentrations*

| Salinity sources and concentrations (dS $m^{-1}$) |
|---|
| Basic nutrient solution (BNS) = 2.50 dS $m^{-1}$ |
| NaCl (50 mM) + BNS (2.50 dS $m^{-1}$) = 7.13 dS $m^{-1}$ |
| $KNO_3$ (50 mM) + BNS (2.50 dS $m^{-1}$) = 8.55 dS $m^{-1}$ |
| High strength nutrient solution (NS) = 7.13 dS $m^{-1}$ |

(Zn) and 0.048 molybdenum (Mo). The second treatment was prepared by adding 50 mM NaCl salt with 2.5 dS $m^{-1}$ BNS, while the third treatment used a mixture of 50 mM $KNO_3$ fertilizer with 2.5 dS $m^{-1}$ BNS. The fourth treatment was done by increasing the concentration of BNS from 2.5 dS $m^{-1}$ to 7.13 dS $m^{-1}$. Each treatment solution was prepared in 200 L fertilizer containers, which were checked and quantified using electrical conductivity (EC) meter (Model HI-98311, Hanna Instrument, USA).

## Plant Maintenance and Treatment Applications

Seeds were sown in a germination tray filled with 100% peat moss and placed under 25% shade on a 1.2-meter bench. Eight days after sowing (DAS), the uniform-sized seedlings were selected and transplanted into a 400 ml pot filled with 100% cocopeat for grafting. All the seedlings designated as rootstock were daily watered by manual drench. Rock melon seedlings in germination tray sown as scion were maintained and watered at field capacity daily. At 13 DAS, all the uniformed-sized scion and rootstock were selected and grafted together using the tongue approach grafting (TAG) technique, a procedure described by Lee and Oda (2003). At six and 12 days after grafting (DAG), approximately 0.5 g of N: P: K (15:15:15) compound fertilizer were given to all grafted plants and daily watered up to field capacity. At 18 DAG, uniform sizes grafted plants were transplanted into the 12 liters of white polyethylene bags filled with 100% cocopeat.

As the plant grew, excess water shoots were removed to increase the growth of the main shoot. The growing shoots were attached to a rope to support the plant's structure and facilitate maintenance. During the reproductive stage, assisted pollination was done from 0830 h to 1030 h. Male flowers were attached to female flowers with the ratio of flowers used at 3:1; male: female to initiate the pollination process. Pollinated flowers were labeled with the date and time. Approximately two to three female flowers per plant were pollinated along the flowering stages. At the fruit setting stage, only one fruit per plant was maintained throughout this experiment, whereas the rest was removed. Growing fruits were supported with the rope to prevent abortion. Pest and disease management was done when necessary, depending on the growing stages

of the plants. At 70 days after transplantation (DAT), all the fruits were harvested with careful handling for data collection.

At 18 DAG, the solution of the treatments was manually drenched using plastic cups with the amounts of 1 liter/plant for 70 days. The frequency of the treatment solution given was increased gradually according to the growing stages as once a day (1-5 DAT), twice/day (6-15 DAT), thrice/day (16-35 DAT), fourfold/day (36-55 DAT), and fivefold/day (56–70 DAT). The EC of the growing media was determined using the pour-through method (Cavins et al., 2000) at vegetative (15 DAT) and fruiting (50 DAT) stages from 1300 h to 1400 h. The EC of four treatment solutions recorded were 2.73, 8.62, 9.25, and 9.05 dS $m^{-1}$ for BNS, NaCl + BNS, $KNO_3$ + BNS, and high strength NS, respectively. During the experiment, average maximum temperature and relative humidity were recorded once a week at 1400 h under natural photoperiod conditions (12 hours light/12 hours dark). The maximum temperature recorded was 35.0 (±5) ºC, with relative humidity (RH) at 62.4 (±10) %. Besides, average carbon dioxide concentrations and light intensity were recorded once a week as 459.9 ppm and 986.17 µmol $m^{-2}s^{-1}$, respectively.

## Data Collection

**Growth Measurements.** Plants were randomly sampled from each treatment to determine plant height, stem diameter, leaf number, total leaf area, and dry weight. Dry weight components, including leaf, stem, and root, were taken at 70 DAT. Plant height was measured from the graft union to the highest shoot tip using a measuring tape. Scion diameter was measured at 1 cm from the growing media surface using an electronic digital caliper (Model CD6''CS Mitutoyo Corp., Japan). At the same time, the leaf number was manually counted based on fully expanded leaves. The whole plants were then harvested and separated into leaf, stem, and root to determine the leaf areas and dry weight matter. Leaf areas were measured and recorded as total leaf area per plant using an automatic leaf area meter (Model LI-3100C, LI-COR Biosciences, USA). All samples were dried to constant weight for at least 72 hours in a forced draught oven at 70 °C before being weighed using a digital analytical balance (Model CDS125, Mitutoyo Inc., Japan).

## Physiological Process

**Relative Water Content (RWC).** The water status of the plants was determined by RWC in the leaves using Khare et al.'s (2010) method. RWC was measured at 55 DAT on a fully expanded leaf. Samples of leaves were kept in the icebox and were carried to the laboratory. Ten leaf discs of 5 mm diameter were cut using a single hole puncher, and the fresh weight (FW) was recorded using a digital analytical balance. The leaf discs were then floated in a small dish containing deionized water for four hours to regain turgidity and reweighted to estimate the turgid weight (TW). Later, the leave discs were dried in a drying oven

at 70 °C for 72 hours to determine the dry weight (DW). The RWC was calculated based on the following equation, and the values were expressed in percentage:

RWC (%) = (FW – DW / TW – DW) x 100%

where,
RWC = Relative water content
FW = Fresh weight
DW = Dry weight
TW = Turgid weight

**Leaf Gas Exchanges.** Leaf gas exchanges were determined by measuring the net photosynthesis, stomatal conductance, and transpiration rate on a selected fully expanded leaf at 55 DAT. The measurements were taken using a portable close photosynthesis machine (infra-red gas analyzer, Li 6400, LI-COR Biosciences, USA) between 9.30 a.m. to 10.30 a.m. with three measurements for each leaf. The measurements used optimal cuvette conditions, at 1000 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD), 400 µmol/ mol carbon dioxide ($CO_2$) at 30 °C cuvette temperature, and 60% relative humidity with the air flow rate set at 500 $cm^3$/min. Irradiance was provided by a light emitting diode red, green, and blue (LED RGB) light source (LI-6400-02B, LI-COR Biosciences, USA).

**Maximum Efficiency of Photosystem II ($F_v/F_m$).** The chlorophyll fluorescence measurements were taken on a selected fully expanded leave at 55 DAT. Chlorophyll fluorescence was measured using a portable fluorescence spectrometer (Mini-PAM, WALZ, Germany). Before the measurements started, the leaves' surface was attached to a light-exclusion clip for 20 minutes. The leaf clip shutter plate was then slid to the open position, and the exposed leaf area was illuminated on the sensor head. As a result, the chlorophyll fluorescence was expressed in $F_v/F_m$, where $F_v$ is variable fluorescence and $F_m$ is maximal fluorescence (Lambers et al., 2008).

**Relative Chlorophyll Content (SPAD) and Photosynthetic Pigments.** Relative chlorophyll content was measured on the fully expanded leaves of each plant at 55 DAT using a leaf chlorophyll meter (SPAD-502 Plus Chlorophyll Meter, Konica Minolta, Inc., Japan). The measurements were taken from three different spots on the leaf surface. The measurements of photosynthesis pigments were taken at 55 DAT. Photosynthetic pigments consisted of chlorophyll *a*, *b*, total chlorophyll (*a* + *b*), and chlorophyll *a*/*b* ratio. Three plant samples of fully expanded leaves were selected from each replication. Samples were taken from the leaf samples using a single-hole puncher at a 5 mm diameter. After a modified procedure, pigments were extracted from ten leaf disks using dimethyl sulfoxide (Nikolopoulos et al., 2008). First, samples were pipetted with 10 ml of dimethyl sulfoxide and then incubated at 65 °C in an oven for four hours until all the pigments were extracted and the leaf disks became transparent. Then, a 3 ml aliquot of the green color was pipetted into the cuvette, and 3 ml of dimethyl sulfoxide was pipetted into another cuvette to serve as a blank. Samples were quantified using a

spectrophotometer (OPTIZEN™ POP UV Vis Spectrophotometer, Korea) and were read at 649, 665, 480, and 510 nm under low light conditions. Chlorophylls and total carotenoid content were calculated based on the following equations (Lichtenthaler & Buschmann, 2001):

nmol (Chl *a*) / cm$^2$ = [(12.47 $E_{665}$ – 3.62 $E_{649}$) x V x 1.119] / A

nmol (Chl *b*) / cm$^2$ = [(25.06 $E_{649}$ – 6.45 $E_{665}$) x V x 1.102] / A

nmol (Chl *a* + *b*) / cm$^2$ = (Chl *a*) + (Chl *b*)
(Chl *a*/*b*) = Chl *a* / Chl *b*

nmol (Carotenoid) / cm$^2$ = [(7.60$E_{480}$ - 1.49$E_{510}$) x V x 1.102] / A

where,
V = Final volume of aliquot
A = Total area in m$^2$ of the leaf tissue extracted
E = Absorbance of aliquot

*Note*. Chlorophyll *a*, *b*, total chlorophyll, and total carotenoid content were expressed as nmole/cm$^2$ of FW materials, while chlorophyll *a*/*b* is a dimensionless ratio.

## Yield Components

Fruit yield components consisted of fruit weight and fruit retention time. At 70 DAT, the fruit was harvested and weighed using a digital analytical balance. The fruit retention time was calculated based on the total days of the fruit retained on the stem that started on the day of assisted pollination until harvesting day.

## Fruit Quality Components

Fruit quality is referred to as its chemical characteristics as the measurements consisted of pH, total soluble solid (TSS), total titratable acidity (TTA), sugar acid ratio, vitamin C, and fruit firmness. Harvested fruit was then cut, and the juice was extracted and transferred into a digital refractometer (PR-100SA, Atago CO., LTD., Japan), and the reading was taken in degrees Brix ($^o$Bx). Determination of vitamin C was done using the volumetric methods of titration according to Pisoschi et al. (2009). The pulp was blended, and 10 g of samples were mixed with 20 ml of 3% phosphoric acid ($HPO_3$) (Sigma-Aldrich, USA) and filtered. Then, 10 ml of the filtrate was pipetted and titrated against dichlorophenol indophenol (DCPIP) (Systerm Chemicals, Malaysia) until the solution turned slightly pink. Another 5 g of the blended samples were mixed with 50 ml of distilled water for pH and TTA determinations. The TTA was quantified as the methods described by Melkamu et al. (2009). Both pH and TTA were read using a titrator instrument (Metrohm 848 Titrino plus, Germany). The value for TTA was expressed by citric acid, which served as a major organic acid. The sugar-acid ratio was calculated by the dimensionless ratio of TSS/TTA. Fruit firmness was measured using a texture analyzer (TA.XT Plus 100, United Kingdom). A cylinder probe of 5 mm diameter size was forced onto the pulp surface, and the reading was expressed in Newton (N).

**Organoleptic Assessment**

Vallone et al.'s (2013) method did a sensory evaluation for rock melon pulp. The evaluation involved 16 untrained panelists, who were given six pieces of ripe fruits from each treatment. The fruits used were harvested two hours before the beginning of the test. The panelists were requested to assess the fruits' color, sweetness, texture, and flavor, where the scores were based on a scale from zero (unacceptable) to seven points (perfect).

**Statistical Analyses**

All the data taken was computed using a statistical analysis system (SAS) (version 9.4). All the variables were assessed for normal distribution using a univariate procedure. Variables were not meet the normally distributed curve were transformed using log transformation. The general linear model (GLM) procedure was used for variance (ANOVA) analysis, and mean comparisons at $P \leq 0.05$ were done using Duncan's Multiple Range Test (DMRT). Relationships among the variables for all salinity sources treatments were pooled and determined using Pearson's correlation coefficients ($r$) at $P \leq 0.05$ by correlation procedure. The data for fruit sensory evaluation taken by 16 panelists was assessed using the GLM procedure and the mean comparison test using orthogonal contrast at $P \leq 0.05$.

## RESULTS

**Effect of Salinity Sources on Growth**

The growth measurements include stem diameter, leaf number, total leaf area, leaf, and stem dry weight of grafted rock melon were significantly affected ($P < 0.05$) by salinity sources (Table 2).

Table 2

*Effects of salinity sources on growth as plant height, stem diameter, leaf number, total leaf area, leaf, stem, and root dry weight of grafted rock melon (Mean ± SD; n=4)*

| Factor | Levels | Plant height (cm) | Stem diameter (mm) | Leaf number | Total leaf area (cm²) | Leaf DW (g) | Stem DW (g) | Root DW (g) |
|---|---|---|---|---|---|---|---|---|
| Salinity sources | BNS | 224.7 ± 7.8[a] | 10.39 ± 0.42[b] | 35.2 ± 2.56[ab] | 12810.9 ± 296.63[a] | 83.652 ± 15.21[a] | 37.015 ± 5.58[a] | 6.99 ± 2.69[a] |
| | NaCl + BNS | 223.6 ± 6.39[a] | 10.07 ± 0.30[bc] | 35.6 ± 1.45[ab] | 9909.0 ± 779.29[b] | 52.264 ± 8.27[b] | 29.956 ± 5.55[b] | 6.028 ± 1.68[a] |
| | $KNO_3$ + BNS | 226.5 ± 5.54[a] | 11.39 ± 0.38[a] | 32.9 ± 0.88[b] | 12284.7 ± 1121.88[a] | 59.699 ± 10.8[b] | 42.044 ± 7.26[a] | 7.208 ± 3.13[a] |
| | High strength NS | 230.8 ± 4.04[a] | 9.74 ± 0.19[c] | 37.7 ± 1.92[a] | 9517.2 ± 657.94[b] | 51.859 ± 5.85[b] | 25.995 ± 3.30[b] | 6.834 ± 5.08[a] |

*Note*. Means in each column with different letters within each level indicate significant differences at a 5% level of significance according to Duncan's Multiple Range Test (DMRT). BNS = Basic nutrient solution; NS = Nutrient solution; DW = Dry weight

Salinity induced by $KNO_3$ + BNS significantly increased stem diameter compared to BNS, NaCl + BNS, and high-strength NS with the respective increments of 8.78%, 11.59%, and 14.49%. However, this treatment application significantly reduced leaf number compared to high-strength NS, resulting in 12.73% reductions. In total leaf area measurements, salinity induced by $KNO_3$ + BNS was similar to control, while significantly higher than NaCl + BNS and high strength NS applications with their respective increments of 19.34% and 22.53%. Leaf dry weight was significantly reduced by NaCl + BNS, $KNO_3$ + BNS, and high strength NS applications compared to control with their respective reductions of 37.52%, 28.63%, and 38.01%. In addition, dry stem weight was significantly reduced by NaCl + BNS and high strength NS applications compared to BNS with their respective reductions of 19.07% and 29.77%.

**Effect of Salinity Sources on Physiological Process**

The relative water content of grafted rock melon was not significantly affected ($P > 0.05$) by salinity sources (Table 3). Therefore, it is indicated that saline treatments have shown comparable water status with BNS.

Table 3

*Effects of salinity sources on the relative water content of grafted rock melon (Mean ± SD; n=4)*

| Factor | Levels | Relative water content (%) |
|---|---|---|
| Salinity sources | BNS | 80.77 ± 3.04[a] |
| | NaCl + BNS | 74.61 ± 4.22[a] |
| | $KNO_3$ + BNS | 76.47 ± 2.75[a] |
| | High strength NS | 76.15 ± 2.39[a] |

*Note*. Means in each column with different letters within each level indicate significant differences at a 5% level of significance according to Duncan's Multiple Range Test (DMRT). BNS = Basic nutrient solution; NS = Nutrient solution

Chlorophyll fluorescence and all leaf gas exchange parameters taken, such as net photosynthesis, stomatal conductance, and transpiration rate in grafted rock melon, were not significantly affected ($P > 0.05$) by salinity sources (Table 4).

Table 4

*Effects of salinity sources on leaf physiology as net photosynthesis, stomatal conductance, transpiration rate, and chlorophyll fluorescence of grafted rock melon (Mean ± SD; n=4)*

| Factor | Levels | Net photosynthesis (mol $CO_2$ $m^{-2}s^{-1}$) | Stomatal conductance (mol $H_2O$ $m^{-2}s^{-1}$) | Transpiration rate (mmol $H_2O$ $m^{-2}s^{-1}$) | Chlorophyll fluorescence ($F_v/F_m$) |
|---|---|---|---|---|---|
| Salinity sources | BNS | 12.103 ± 2.64[a] | 0.263 ±0.10[a] | 3.817 ± 0.91[a] | 0.783 ± 0.02[a] |

Table 4 *(Continue)*

| Factor | Levels | Net photosynthesis (mol $CO_2$ $m^{-2}s^{-1}$) | Stomatal conductance (mol $H_2O$ $m^{-2}s^{-1}$) | Transpiration rate (mmol $H_2O$ $m^{-2}s^{-1}$) | Chlorophyll fluorescence ($F_v/F_m$) |
|---|---|---|---|---|---|
| Salinity sources | NaCl + BNS | 10.627 ± 0.54[a] | 0.166 ± 0.04[a] | 2.807 ± 0.40[a] | 0.788 ± 0.02[a] |
| | $KNO_3$ + BNS | 10.723 ± 1.13[a] | 0.189 ± 0.03[a] | 3.069 ± 0.39[a] | 0.771 ± 0.03[a] |
| | High strength NS | 12.635 ± 2.24[a] | 0.242 ± 0.10[a] | 3.609 ± 1.07[a] | 0.777 ± 0.05[a] |

*Note*. Means in each column with different letters within each level indicate significant differences at a 5% level of significance according to Duncan's Multiple Range Test (DMRT). BNS = Basic nutrient solution; NS = Nutrient solution

Moreover, all photosynthetic pigments measured in grafted rock melon were significantly affected ($P < 0.05$) by salinity sources, while no significant effect ($P > 0.05$) was observed in relative chlorophyll content in the SPAD unit (Table 5).

Table 5

*Effects of salinity sources on relative chlorophyll content (SPAD), chlorophyll* a, b, *total chlorophyll, chlorophyll* a/b *ratio, and carotenoid content of grafted rock melon (Mean ± SD; n=4)*

| Factor | Levels | SPAD | Chl *a* (mg $g^{-1}$) | Chl *b* (mg $g^{-1}$) | Chl *a* + *b* (mg $g^{-1}$) | Chl *a*/*b* (mg $g^{-1}$) | Carotenoid (mg $g^{-1}$) |
|---|---|---|---|---|---|---|---|
| Salinity sources | BNS | 63.7 ± 2.89[a] | 65.27 ± 1.70[a] | 18.29 ± 0.18[b] | 83.56 ± 2.12[a] | 3.57 ± 0.34[a] | 29.25 ± 0.52[a] |
| | NaCl + BNS | 62.2 ± 2.27[a] | 58.50 ± 3.67[a] | 16.98 ± 0.19[b] | 75.48 ± 2.50[a] | 3.45 ± 0.23[a] | 27.08 ± 1.37[a] |
| | $KNO_3$ + BNS | 58.1 ± 1.57[a] | 13.52 ± 3.64[b] | 43.39 ± 0.17[a] | 56.86 ± 3.82[b] | 0.32 ± 0.58[b] | 20.86 ± 0.51[b] |
| | High strength NS | 65.2 ± 1.46[a] | 64.02 ± 5.87[a] | 18.71 ± 0.16[b] | 82.72 ± 3.05[a] | 3.42 ± 0.15[a] | 29.12 ± 1.03[a] |

*Note*. Means in each column with different letters within each level indicate significant differences at a 5% level of significance according to Duncan's Multiple Range Test (DMRT). BNS = Basic nutrient solution; NS = Nutrient solution; Chl = Chlorophyll

Salinity induced by $KNO_3$ + BNS application significantly decreased chlorophyll *a* compared to BNS, NaCl + BNS, and high strength NS with the respective reductions of 79.29%, 76.89%, and 78.89%. In contrast, chlorophyll *b* was significantly increased by $KNO_3$ + BNS application compared to BNS, NaCl + BNS,

and high strength NS with the respective increments of 57.85%, 60.87%, and 56.88%. Total chlorophyll was significantly decreased by $KNO_3$ + BNS application compared to control, NaCl + BNS, and high strength NS with the respective reductions of 31.95%, 24.67%, and 31.26%. The chlorophyll *a*/*b* ratio was significantly decreased by $KNO_3$ + BNS application compared to BNS, NaCl + BNS, and high strength NS with the respective reductions of 91.04%, 90.72%, and 90.64%. Carotenoid was significantly decreased by $KNO_3$ + BNS application than BNS, NaCl + BNS, and high strength NS with the respective reductions of 29.70%, 22.97%, and 28.37%.

### Effect of Salinity Sources on Yield Components

Salinity source applications significantly affected ($P < 0.05$) both yield components in fruit retention time and fruit weight for grafted rock melon (Table 6).

Table 6

*Effects of salinity sources on yield components such as fruit retention time and fruit weight of grafted rock melon (Mean ± SD; n=4)*

| Factor | Levels | Fruit retention time (Day) | Fruit weight (kg) |
|---|---|---|---|
| Salinity sources | BNS | 51.5 ± 0.78[a] | 1.84 ± 2.66[a] |
| | NaCl + BNS | 46.2 ± 1.78[c] | 1.31 ± 1.90[b] |
| | $KNO_3$ + BNS | 43.1 ± 1.04[d] | 1.32 ± 1.64[b] |
| | High strength NS | 48.1 ± 1.53[b] | 1.33 ± 3.92[b] |

*Note*. Means in each column with different letters within each level indicate significant differences at a 5% level of significance according to Duncan's Multiple Range Test (DMRT). BNS = Basic nutrient solution; NS = Nutrient solution

Salinity induced by NaCl + BNS, $KNO_3$ + BNS, and high strength NS applications significantly decreased fruit retention time than those grown in control, with their respective reductions of 10.29%, 16.31%, and 6.6%. Similarly, fruit weight was significantly decreased by NaCl + BNS, $KNO_3$ + BNS, and high strength NS applications than those grown in control with their respective reductions of 28.80%, 28.26%, and 27.72%.

### Effect of Salinity Sources on Fruit Quality Components

Salinity source applications significantly affected ($P < 0.05$) fruit quality components of grafted rock melon as total soluble solid, sugar acid ratio, and firmness (Table 7).

Table 7

*Effects of salinity sources on fruit quality such as pH, total soluble solid, total titratable acidity, sugar acid ratio, vitamin C, and firmness of grafted rock melon (Mean ± SD; n=4)*

| Factor | Levels | pH | Total soluble solid (ºBx) | Total titratable acidity (%) | Sugar acid ratio (TSS/TTA) | Vitamin C (mg/100g FW) | Firmness (N) |
|---|---|---|---|---|---|---|---|
| Salinity sources | BNS | 6.56 ± 0.48[a] | 13.9 ± 3.16[b] | 0.155 ± 0.12[a] | 89.61 ± 3.64[c] | 0.35 ± 0.03[a] | 7.742 ± 1.57[b] |
| | NaCl + BNS | 6.72 ± 0.42[a] | 15.4 ± 2.95[a] | 0.136 ± 0.15[a] | 114.10 ± 2.36[a] | 0.39 ± 0.05[a] | 10.919 ± 1.14[a] |
| | $KNO_3$ + BNS | 6.62 ± 0.30[a] | 14.0 ± 3.02[b] | 0.155 ± 0.15[a] | 93.05 ± 5.73[bc] | 0.38 ± 0.03[a] | 12.201 ± 0.75[a] |
| | High strength NS | 6.72 ± 0.47[a] | 15.6 ± 3.27[a] | 0.141 ± 0.18[a] | 111.78 ± 2.89[ab] | 0.38 ± 0.04[a] | 10.104 ± 1.01[ab] |

*Note*. Means in each column with different letters within each level indicate significant differences at a 5% level of significance according to Duncan's Multiple Range Test (DMRT). BNS = Basic nutrient solution; NS = Nutrient solution; FW = Fresh weight

Total soluble solid was significantly increased by NaCl + BNS and high strength NS applications compared to BNS with their respective increment of 9.74% and 9.09%. In addition, the sugar-acid ratio was significantly increased by NaCl + BNS compared to BNS and $KNO_3$ + BNS, respectively, with 21.91% and 18.45%. At the same time, firmness was significantly increased by NaCl + BNS and $KNO_3$ + BNS compared to BNS with their respective increment of 29.1% and 36.55%.

Based on the fruit preferences score, the sweetness and flavor of the fruit were significantly affected ($P < 0.01$) by the comparisons between BNS and $KNO_3$ + BNS, NaCl + BNS, and $KNO_3$ + BNS as well as $KNO_3$ + BNS and high strength NS (Table 8).

Table 8

*Sensory evaluation of rock melon grown at different salinity sources*

| Salinity sources | Fruit preferences score | | |
|---|---|---|---|
| | Sweetness | Texture | Flavor |
| BNS | 6.07 | 5.71 | 6.29 |
| NaCl + BNS | 6.43 | 5.79 | 6.14 |
| $KNO_3$ + BNS | 3.93 | 5.14 | 4.79 |
| High strength NS | 6.00 | 5.71 | 5.93 |

Table 8 *(Continue)*

| Salinity sources | Fruit preferences score | | |
|---|---|---|---|
| | Sweetness | Texture | Flavor |
| *Paired orthogonal contrast* | | | |
| BNS vs. NaCl + BNS | ns | ns | ns |
| BNS vs. $KNO_3$ + BNS | ** | ns | ** |
| BNS vs. High strength NS | ns | ns | ns |
| NaCl + BNS vs. $KNO_3$ + BNS | ** | ns | ** |
| NaCl + BNS vs. High strength NS | ns | ns | ns |
| $KNO_3$ + BNS vs. High strength NS | ** | ns | ** |

*Note*. **Significant at 1% probability level, ns = Not significant
The number of panelists = 16. The maximum possible score is 7
BNS = Basic nutrient solution; NS = Nutrient solution

On the other hand, the sweetness and flavor of the fruit were not significantly affected ($P > 0.05$) by the comparisons between BNS and NaCl + BNS, BNS, and high strength NS and NaCl + BNS and high strength NS.

**Correlation Analysis on Growth, Yield, and Fruit Quality of Grafted Rock Melon**

Table 9 shows the relationships among the selected significant parameters, including growth, yield components, and fruit quality. All the relationships were elaborated based on significant relationships observed towards fruit yield and quality elements.

Table 9

*Pearson's linear correlation coefficients (r) between growth parameters, yield components, and fruit quality of grafted rock melon*

| | SD | LN | TLA | LDW | SDW | FW | FRT | TSS | SAR | FN |
|---|---|---|---|---|---|---|---|---|---|---|
| SD | 1 | -0.50* | 0.64** | 0.32ns | 0.79** | 0.01ns | -0.55* | -0.66** | -0.52* | 0.46ns |
| LN | | 1 | -0.40ns | 0.02ns | -0.27ns | -0.01ns | 0.32ns | 0.59* | 0.18ns | -0.14ns |
| TLA | | | 1 | 0.79** | 0.86** | 0.28ns | 0.11ns | -0.72** | -0.81** | -0.04ns |
| LDW | | | | 1 | 0.64** | 0.38ns | 0.49ns | -0.46ns | -0.68** | -0.32ns |
| SDW | | | | | 1 | -0.04ns | -0.23ns | -0.54* | -0.68** | 0.24ns |
| FW | | | | | | 1 | 0.58* | -0.47ns | -0.14ns | -0.56* |
| FRT | | | | | | | 1 | 0.01ns | -0.09ns | -0.86** |
| TSS | | | | | | | | 1 | 0.61* | 0.14ns |
| SAR | | | | | | | | | 1 | -0.01ns |
| FN | | | | | | | | | | 1 |

*Note*. SD = Stem diameter (mm); LN = Leaf number; TLA = Total leaf area ($cm^2$); LDW = Leaf dry weight (g); SDW = Stem dry weight (g); FW = Fruit weight (kg); FR = Fruit retention time (Day); TSS = Total soluble solid; SAR = Sugar acid ratio; FN = Firmness (N)
**Significant at 1% level of significance; *Significant at 5% level of significance; ns = Not significant

In terms of relationships between growth parameters and yield components, stem diameter was negatively correlated with fruit retention time. There was significant medium negative correlation ($r$ = -0.55; $P \leq 0.05$) between stem diameter and fruit retention time.

Most growth parameters, such as stem diameter, leaf area meter, and stem dry weight, were negatively correlated with fruit quality components, such as total soluble solid and sugar-acid ratio. Among the relationships, the strongest correlation was observed between total leaf area with both fruit quality components, such as total soluble solid ($r$ = -0.72; $P \leq 0.01$) and sugar-acid ratio ($r$ = -0.81; $P \leq 0.01$).

Other than that, relationships between fruit yield and fruit quality components showed that fruit weight and fruit retention time were negatively correlated with fruit firmness. Among the relationships, the strongest negative correlation was observed between fruit retention time and fruit firmness ($r$ = -0.86; $P \leq 0.01$).

## DISCUSSION

### Effect of Salinity Sources on Growth

At the fruit development stage (70 DAT), the growth performance of plants treated under $KNO_3$ + BNS application improved by exhibiting higher stem diameter comparable with total leaf area and dry weight compared to BNS. An increase in growth elements might be attributed to an increase in cell division and cell elongation, which is related to the mineral ion compositions of the plants. Generally, nitrogen and potassium exist in $KNO_3$ and play an important role in plant growth and development. Nitrate is an important ingredient in $KNO_3$ that plays a role in stimulating the development of the plant by synthesizing amino acids and protein (Liu et al., 2014). Besides, potassium is known to improve protein and carbohydrate synthesis, with photosynthates translocated from the leaves (source) to the place where they may be used or stored (sink) (Haddad et al., 2016). Thus, both salt types, like nitrate and potassium, promoted the grafted rock melon to attain high dry matter components.

Al-Hamzawi (2010) has reported that the application of 15 mM $KNO_3$ considerably improved growth parameters by increasing plant height, leaves number, and total leaf area of cucumber. Supplementary $KNO_3$ under NaCl salt-stressed treatments had increased leaf number, leaf area, stem elongation, and dry matter in grafted citrus (Khoshbakht et al., 2014). Despite the positive effects mentioned in most growth parameters (Table 2), salinity induced by $KNO_3$ + BNS that is prolonged until the fruiting stage has resulted in a lower leaf number. It is probably due to the excess nitrates accumulated in the leaves. An increase in nitrate at a certain level will increase the osmotic concentration, thus leaving the salts behind. As a result, the leaves were dehydrated and appeared to be burnt. It was suddenly wilting and becoming yellow or brown. Therefore, the percentage of the leaves aborting increases, consequently reducing the leaf number. Excess nitrate accumulated in plants if

double the amount of fertilization resulted in deleterious effects on plant growth and yield (Sharifi et al., 2011). Similarly, the growth response of leafy vegetables tested under various concentrations of nitrates was strongly decreased after it accumulated higher in the plant (Chen et al., 2004).

**Effects of Salinity Sources on Photosynthetic Pigments**

The results showed that salinity induced using $KNO_3$ + BNS for prolonged periods until the fruiting stage decreased the components of photosynthetic pigments. Excessive $KNO_3$ accumulated in the leaves had increased the salinity levels, negatively affecting the chlorophyll contents. Chlorophyll reduction under saline stress is a common phenomenon attributed to various factors, including inhibition of chlorophyll biosynthesis caused by the activation of the chlorophyllase enzyme (Noreen & Ashraf, 2009) and membrane deterioration caused by salinity-mediated chlorophyll degradation (Ashraf & Bhatti, 2000). It is also shown that the reduction of salt in the stressed plant chlorophyll has also been regarded as a common indication of oxidative stress (Elsheery & Cao, 2008). It is also corroborated by Noreen and Ashraf (2009), who stated that the reduction of chlorophyll content in pumpkin genotypes might have been due to a salt-induced increase in the activity of the chlorophyll degrading enzyme such as chlorophyllase. Cucumber-treated plants under salt stress at 75 mM had decreased the total chlorophyll, chlorophyll *b*, and chlorophyll *a*/*b* ratio (Shu et al., 2012). The reduction of carotenoid content in the leaves is also due to the long exposure to $KNO_3$ salt. More salt is also known to impact photosynthesis through non-stomatal restrictions, including changes in carotenoid content (L. Zhang et al., 2012).

Long-term exposure to salt stress in young leaves causes a decrease in carotenoid levels, even in halophyte plants (Duarte et al., 2013). This result concurred with research conducted on tomatoes (Gong et al., 2013) and maize (Singh et al., 2008). In contrast, plants treated under $KNO_3$ + BNS had the highest chlorophyll b content. Higher chlorophyll b pigment observed is due to the chlorophyll *a* degradation after salt stress exposure. Chlorophyll *b* is the accessory pigment that collects sunlight before being transported into chlorophyll *a,* commonly known as the principal pigment that captures light for photosynthesis. Therefore, more chlorophyll *b* was necessitated and synthesized to sustain the plant's growth by transmitting the light sources into chlorophyll *a* pigment for photosynthesis.

**Effects of Salinity Sources on Yield**

In terms of yield components, fruit retention time was considerably reduced under all saline treatments compared to BNS. Among the saline treatments tested, $KNO_3$ + BNS application had the lowest fruit retention time, followed by NaCl + BNS and high strength NS. Shorter fruit retention time under saline treatments is due to salinity's adverse effect that strongly impaired grafted rock melon's reproductive growth. In

addition, salinity imposed during flower anthesis, or pollination time, had delayed the fruit set due to flower abortion incidence. Therefore, a shorter fruit retention time was obtained in these treatments. This condition had delayed the time of fruit setting, causing higher fruit bearing shown under saline treatments in Figure 1 (B, C, and D).

Reduction in fruit set under salinity was associated with low pollen fertility by decreasing starch concentration through invertase inhibition and lowing carbon fluxes to the anthers, leading to flower abortion (Sheoran & Saini, 1996). In addition, a decrease in fruit set under a saline environment has also been attributed to stigma receptivity reduction (Khan & Abdullah, 2003). A similar finding was obtained by Ghanem et al. (2009), where flowers aborting percentage were significantly higher, and the fruit setting process was delayed in tomatoes under NaCl salinity treatment. They concluded that the accumulation of toxic ions such as sodium ion ($Na^+$) in the female parts caused a high abortion rate by hampering the pollen germination and its subsequent growth. Previous research on mango shows that the $KNO_3$ application, particularly at a 4% level, was mildly phytotoxic to leaves and inflorescences and resulted in necrotic leaves and extremities of the inflorescence branches (Oosthuyse, 1996).

In addition, the reduction in fruit weight under all saline treatment applications in this study suggested the interference of salinity stress towards fruit development. It is noted that the salinity stress limits the productivity of crops, with adverse effects on crop yield (Munns & Tester, 2008). These might be explained by the fact that high salt levels diminish water potential in plants, resulting in less water flowing into fruit and reducing the fruit expansion rate (Al-Ismaily et al., 2014). Enlargement rate reduction during the exponential phase of fruit growth is particularly vulnerable to ionic and osmotic damages induced by ion accumulation in the plants (Helaly et al., 2017). On the other hand, yield reduction in melon is due to nutritional imbalances produced by the disrupted absorption or distribution of essential mineral elements caused by salinity stress (Del-Amor et al., 1999). De L. Pereira et al. (2017) reported

*Figure 1*. Effect of salinity sources as BNS (A), NaCl + BNS (B), $KNO_3$ + BNS (C), and high strength NS (D) on fruit yield of grafted rock melon at 60 DAT, respectively

[*Note*. BNS = Basic nutrient solution; NS = Nutrient solution]

that the average weight of marketable melon decreased when the solution salinity increased. Freitas et al. (2014) found losses of 11% per dS $m^{-1}$ in melon yield irrigated with high-saline water (EC = 4.5 dS $m^{-1}$). Dias et al. (2018) also found that the melon weight (cv. Néctar) was reduced when solution salinity increased above 3.5 dS $m^{-1}$. This finding was supported by a significant medium-positive correlation (Table 9) observed between fruit weight and fruit retention time, indicating that the fruit weight increased when fruit retention time increased.

**Effects of Salinity Sources on Fruit Quality**

Despite those saline treatments negatively affecting the fruit yield component, the fruit quality characteristics, such as total soluble solid, sugar acid ratio, and fruit firmness, were considerably improved. Compared to BNS, salinity induced by NaCl + BNS and high strength NS applications had better total soluble solid and sugar-acid ratio. It indicates that the fruit produced under the saline treatments is sweeter, with better flavor preferences for fruit consumption. Higher TSS fruit content under high salinity water is presumably caused by a lower mean fruit weight that promotes an increase in the photoassimilate concentration (de L. Pereira et al., 2017). Awang et al. (1993) have concluded that fruit quality enhancement by salinity would relate significantly to fruit water depression, raising the relative amount of dry matter and sugars. The results are consistent with previous studies on melon, which found that the total soluble solids content of melon cultivars rose as irrigation water salinity increased (Zulkarami et al., 2010). Moreover, the highest sugar acid ratio recorded in both saline treatments is associated with total soluble solids. Larger differences between total soluble solid and acid content in the fruit pulp treated under saline treatments resulted in a higher sugar-acid ratio.

A significant medium positive correlation supports it is observed between total soluble solid and sugar-acid ratio ($r$ = 0.61; $P \leq 0.05$) (Table 9), indicating direct relationships were established in this study. Saline environments generally raise TSS and fruit juice acid concentrations. It has been proven by previous studies demonstrated on melon, tomato, sweet pepper, and cucumber. Previous studies on melon (cv. *galia*) revealed that the increased concentrations in nutrient solution and duration of application resulted in increased fruit's TSS and sugar-acid ratio (Del-Amor et al., 1999). A high TSS and sugar-acid ratio was exhibited in both saline treatments that was attributed to smaller growth characteristics as a correlation was established in this study. Total soluble sugar was negatively correlated with total leaf area ($r$ = -0.72; $P \leq 0.01$) and stem dry weight ($r$ = -0.54; $P \leq 0.05$), while the sugar-acid ratio was negatively correlated with total leaf area ($r$ = -0.81; $P \leq 0.01$) and stem dry weight ($r$ = -0.68; $P \leq 0.01$) (Table 9).

Salinity induced by NaCl + BNS and $KNO_3$ + BNS applications increased fruit firmness. Improvements in fruit firmness

could be due to smaller cells with thicker walls in the fruit mesocarp under saline conditions (Ruiz et al., 2015). An increase in fruit firmness is probably due to the chemical compositions, such as TSS, ascorbic acid, and lycopene contents (Abdelgawad et al., 2019). It is proven by the study observed in tomatoes (Del-Amor et al., 1999). It could also suggest that the increase in fruit firmness by NaCl and $KNO_3$ + BNS application is due to low fruit retention time. Lower fruit retention time compared to BNS leads to varying levels of fruit maturity. Thus, the shorter fruit retention time obtained in these treatments reduced the conversion time of dry matter content into starch, resulting in higher fruit firmness. The result was corroborated by a significant negative relationship established between fruit firmness and fruit retention time ($r$ = −0.86; $P \leq 0.01$) (Table 9), indicating that fruit firmness increased as fruit retention time decreased.

Fruit preferences for sweetness, texture, and flavor were represented as total soluble solid, firmness, and sugar-acid ratio from instrumental results. Panelists failed to distinguish the fruits' texture well as greater fruit firmness achieved by NaCl + BNS and $KNO_3$ + BNS applications compared to BNS from the instrumental results. In terms of sweetness and flavor characteristics, fruits produced by plants under BNS, NaCl + BNS, and high-strength NS treatments are perceived as tastier than $KNO_3$ + BNS treatments. Panelists failed to determine the similarity of the sweetness and flavor between BNS and $KNO_3$ + BNS as no significant difference in the total soluble solid and sugar-acid ratio was observed from the instrumental results. Comparable sweetness and flavor characteristics on fruits grown under BNS, NaCl + BNS, and high-strength NS applications, exhibited similar taste levels. It indicated that the panelists failed to appreciate the increase in total soluble solid and sugar-acid ratio content from both treatments (NaCl + BNS and high strength NS) as presented in the instrumental results. Comparable sweetness and flavor preferences between NaCl + BNS and high-strength NS applications by panelists are consistent with the instrumental results.

Based on the overall variables taken, the grafted rock melon exhibited different characteristics depending on the saline treatments. Salinity induced using $KNO_3$ + BNS application could sustain most growth parameters. It can sustain the leaf gas exchange components and relative water content. However, the chlorophyll and carotenoid content were significantly impaired. On the other hand, this treatment reduced the yield component, but the fruit quality has been sustained. Application of NaCl + BNS and high strength NS had similar trends based on overall variables taken in grafted rock melon. Both saline treatments reduced most growth parameters, but all the physiological process was sustained. On the other hand, the yield component was reduced, but the fruit quality was improved better than BNS.

## CONCLUSION

Supplementation of $KNO_3$ salt (50 mM) into nutrient solution showed a higher tendency to increase growth while sustaining fruit quality. However, the physiological process and fruit yield had reduced. Salinity induced by NaCl salt (50 mM) and the high-strength nutrient solution had high fruit quality without interfering with all physiological processes. However, the growth and yield were reduced. Based on overall characteristics evaluated among all saline treatments, incorporation of NaCl (50 mM) + BNS is recommended to be adopted due to its ability to increase fruit quality without interfering with all physiological processes, and it is inexpensive and easily available.

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# *In vitro* Assessment of Multistrain Probiotic on Its Safety, Biofilm Formation Capability, and Antimicrobial Properties Against *Aeromonas hydrophila*

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## ABSTRACT

Probiotics have been increasingly considered an alternative to antibiotics in combating disease outbreaks. Combined probiotics have been studied to possibly harbor synergistic effects that could provide better protection for aquatic species. Three potential probiotics, which had shown *in vitro* antagonism towards *Aeromonas hydrophila* in this study, were *Bacillus amyloliquefaciens* (L9, isolated from the blue swimming crab), *Lysinibacillus fusiformis* (A2, isolated from a microalga), and *Enterococcus hirae* (LAB3, isolated from the Asian seabass) were combined into a probiotic mixture. The probiotic mixture produced significantly higher biofilm ($P < 0.05$) (2.441 ± 0.346) than *A. hydrophila* (0.578 ± 0.124) during 24-h and showed a continuous increase in production at 48-h and 72-h time intervals, respectively. Furthermore, no hemolytic action was observed when the probiotic mixture was streaked on sheep blood agar (5%), whereas *A. hydrophila* presented α-hemolysis. The lowest concentration of the probiotic mixture ($10^7$ CFU $mL^{-1}$) significantly inhibited ($P < 0.05$) the growth of *A. hydrophila* at $10^6$ CFU $mL^{-1}$ after 24 h of incubation, where bacterial count in the treatment was 6.595 ± 0.218 CFU $mL^{-1}$, which was significantly lower ($P < 0.05$) than the control (7.247 ± 0.061 CFU) $mL^{-1}$. Significant reduction ($P < 0.05$) in *Aeromonas* count from 7.532 ± 0.026

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CFU mL$^{-1}$ to 6.883 ± 0.015 CFU mL$^{-1}$ was observed at 12 hours of co-incubation. Hence, this research suggests that the probiotic mixture of L9, A2, and LAB3 potentially confers protection against *A. hydrophila* infection due to their characteristics meeting the criteria of probiotics.

*Keywords*: *Aeromonas hydrophila*, antagonism, biofilm formation, hemolytic activity, probiotic mixture

## INTRODUCTION

Aquaculture is a rapidly expanding multibillion-dollar industry with a compound annual growth rate of 5.3% per year (2001–2018) compared to terrestrial meat production, standing at 2.7% (Food and Agriculture Organization of the United Nations [FAO], 2020). Global aquaculture production reached a record of 114.5 million tonnes in 2018, providing >50% of food fish for human consumption (FAO, 2020). Furthermore, with the human population projected to exceed 9 billion by 2050, farmed fish and shellfish production will need to increase by 133% to meet worldwide fish demands (Duarte et al., 2020).

Aquaculture, also known as aquafarming, produces fish, crustaceans, mollusk, aquatic plants, algae, and other organisms in marine, brackish, and freshwater systems under controlled conditions (FAO, 2020; Naylor et al., 2021). One of the main causes of economic loss in cultured fish is bacterial diseases where under stressful conditions, the pre-existing bacteria invade the host and causes a disease outbreak (Fazio, 2019; Morae & Martins, 2004). Aeromonads such as *A. hydrophila* and *A. veronii* spread horizontally, causing hemorrhagic disease, ulcerative syndrome, and Motile *Aeromonas* Septicemia (MAS) in fish, usually resulting in high mortalities, especially in farmed warm-water fishes (Gudmundsdottir & Bjornsdottir, 2017; Janda & Abbott, 2010). Experimental challenge of *Oreochromis aureus* with *A. hydrophila* revealed massive hemocyte aggregation and cellular necrosis of gills, hepatopancreas, and to a lower extent in the digestive system of infected fish (AlYahya et al., 2018).

For decades, animal disease prevention and treatment have revolved around using chemical additives and veterinary medicines, especially antibiotics. However, reports on the detrimental effects caused by the broad spectrum of chemotherapeutics, such as the emergence of drug-resistant bacteria, potential human health hazards, environmental contamination, and elimination of gut microflora beneficial to fishes, have triggered interest in experimentation with biological and eco-friendly approaches (Akanmu, 2018).

The development of alternative therapies to eradicate bacterial pathogens in animal production is indispensable. Several methods have been successfully tested in the aquaculture industry, particularly the application of probiotics (Cavalcante et al., 2020; Munir et al., 2018). Probiotics are live microorganisms beneficial to the host, which alter the microbial community associated with the host or environment, ensuring better feed utilization, health improvement,

and enhanced disease resistance (Tran et al., 2022; Yilmaz et al., 2022). Most probiotics used as biological control agents in aquaculture are lactic acid bacteria (LAB) (*Lactobacillus*, *Enterococcus*, *Lactococcus*, *Micrococcus*, and *Carnobacterium*) (Román et al., 2012). In addition, other genera or species belonging to the genus *Vibrio* (Restrepo et al., 2021), *Bacillus* (Elsabagh et al., 2018), *Pseudomonas* (Qi et al., 2020), and *Aeromonas* (Jinendiran et al., 2021) were also extensively studied. One such example of probiotics application is the diet supplementation of *Bacillus licheniformis* to *Oreochromis mossambicus*, which showed the potential to reduce ammonia toxicity, improve growth performance, general health status as well as resistance to a pathogen (*A. hydrophila*) (Gobi et al., 2018; Gopi et al., 2022). However, many studies only emphasized the different health effects produced by single-strain probiotics, such as *Bacillus subtilis* in the red hybrid tilapia (*Oreochromis* sp.) (Ng et al., 2014) and *Lactobacillus plantarum* in the Nile tilapia (*Oreochromis niloticus*) (Gewaily et al., 2021).

Nevertheless, the supplementation of multi-strain probiotics (MSPs) is relatively new. The combination of probiotics containing *Bacillus subtilis* E20, *Lactobacillus pentosus* BD6, *Saccharomyces cerevisiae* P13, and *Lactobacillus fermentum* LW2 was observed to improve the growth performance and health status of the Asian seabass (*Lates calcarifer*) (Lin et al., 2017). The efficacy of MSPs is due to symbiosis caused by the positive interrelationship between candidate strains (Puvanasundram et al., 2021). A previous study highlighted the benefits of supplementing mixed probiotics (combining *Lysinibacillus fusiformis* SPS11, *Bacillus amyloliquefaciens* L9, and *Enterococcus hirae* LAB3) to improve biofloc production in red hybrid tilapia culture (Zabidi, Yusoff, et al., 2021). Another study showed mixed probiotics (*L. fusiformis* SPS11, *L. fusiformis* A2, and *Bacillus megaterium* I24) protected *Artemia* against *Vibrio alginolyticus* infection (Chean et al., 2021). Thus, the present study aimed to determine whether the probiotic mixture (containing *B. amyloliquefaciens* L9, *L. fusiformis* A2, and *E. hirae* LAB3) was selected based on a series of *in vitro* antagonistic assays and could potentially eliminate *A. hydrophila* and can ultimately be categorized as a beneficial probiotic mixture. Additionally, the biofilm formation capability of the probiotic mixture was also assessed in this study.

## MATERIALS AND METHODS

### Probiotic Strains

The agar and broth media used in this study were Trypto-casein soy agar (TSA) (Biokar Diagnostics, France) and Trypto-casein soy broth (TSB) (Biokar Diagnostics, France), respectively. In addition, potential probiotics used in this study were isolated and identified through earlier research at the Laboratory of Fish Health, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia (Table 1).

Table 1
*Potential probiotics isolated from different hosts*

| Probiotic | Strain | Genbank accession number | Host | Reference |
|---|---|---|---|---|
| *Bacillus amyloliquefaciens* | L9 | MN096656 | Blue swimming crab (*Portunus pelagicus*) | Azrin et al. (2019) |
| *Enterococcus hirae* | LAB3 | MK757970 | Asian seabass (*Lates calcarifer*) | Masduki et al. (2020) |
| *Lysinibacillus fusiformis* | A2 | MK764895 | Microalga (*Amphora* sp.) | Rosland et al. (2021) |

All isolates were cultured overnight in the TSB medium before any screening assays. *Aeromonas hydrophila*, provided by Laboratory of Aquatic Animal Health and Therapeutics, Institute of Biosciences, Universiti Putra Malaysia, was previously isolated from diseased *Oreochromis* sp. A single colony from each potential isolate and *A. hydrophila* was picked and inoculated separately into the TSB medium and incubated at 30 °C for 24 h.

### Antagonistic Screening of Potential Probiotics and Mixture

All potential probiotics were screened *in vitro* on TSA agar to test for inhibitory effects against *A. hydrophila*. Overnight cultures of probiotics and the pathogen were centrifuged at 1,957 × *g* for 10 min. The supernatant was discarded, and cells were resuspended with distilled water (dd$H_2O$). The absorbance of the resuspended overnight culture of *A. hydrophila* was measured using the UV Spectrophotometer (Eppendorf, Germany) at 550 nm to adjust the concentration of the bacterial culture.

The agar well diffusion assay was used as a primary screening step to determine the antagonism of potential probiotics towards *A. hydrophila*. The assay was conducted on TSA agar using isolates cultured overnight in TSB following Rengpipat et al. (2008), with some modifications. After 24 h, the pathogenic *A. hydrophila* was diluted to the concentration of $10^6$ CFU $mL^{-1}$ and swabbed onto the TSA agar using a sterile cotton swab. Next, the agar was punched with a 5 mm sterile cork borer, and 10 µL of the overnight culture of potential probiotics ($10^9$CFU $mL^{-1}$) was inoculated into the well. The plate was then incubated at 30 °C for 24 h, and the inhibition zones were observed while measuring the diameter in mm. Furthermore, an equal volume (1:1:1) of the probiotics was added to a 1.5 mL microcentrifuge tube to produce a probiotic mixture. The probiotic mixture was then incubated for 20 min at 30 °C. The antagonistic activity of the probiotic mixture against *A. hydrophila* was also evaluated as described above.

### Compatibility Assay of the Selected Potential Probiotics

The compatibility among selected probiotics was also evaluated using the agar well diffusion assay (Rengpipat et al., 2008). The probiotics combined into a probiotic mixture were cultured overnight in TSB prior to the assay. A sterile cotton swab was used for each probiotic to obtain the culture before spreading it onto the TSA. Afterward, a 5 mm cork borer was used to punch two wells on the swabbed agar to inoculate the other two probiotics. These steps were repeated for each probiotic in triplicate. Finally, the plates were incubated for 24 h at 30 °C, and the presence of any inhibitory zones was duly recorded.

### *In vitro* Hemolysis Assessment

Sheep blood agar plates (5%) (Thermo Scientific Microbiology Sdn. Bhd., Malaysia) were used to detect the presence of hemolysin in the hemolysis test to rule out potential pathogenicity. This assay determines whether the probiotic mixture (comprising *B. amyloliquefaciens* L9, *L. fusiformis* A2, and *E. hirae* LAB3) can produce enzymes that destroy red blood cells if supplemented with a host. Overnight cultures of the potential probiotic mixture and *A. hydrophila* (positive control) were streaked onto two separate blood agar plates and incubated overnight for 30 °C. After 24 h, the results of both blood agars were compared, and the presence or absence of clearing zones around the colonies was observed to interpret the hemolytic activity. Isolates that caused no change in the agar around the colonies were considered non-hemolytic (γ-hemolysis), and isolates showing a clean zone around colonies were considered to be hemolytic (β-hemolysis) (Foulquié Moreno et al., 2003).

### Biofilm Production Assay

With some modifications, the biofilm production assay was conducted following (Bruhn et al., 2007). Before conducting the assay, the potential probiotics and *A. hydrophila* were cultured overnight in the TSB medium on an orbital incubator shaker (BioSan Laboratories Inc., Latvia). The biofilm production assay was performed in triplicate for each sample: the control (TSB only), *A. hydrophila* only, probiotic mixture only, and the three selected single probiotics during 6-, 12-, 24-, 48-, and 72-h, respectively. Initially, 2 mL of TSB media was added into separate glass vials, followed by 200 µL of each bacterial culture at a concentration of $10^9$ CFU $mL^{-1}$. The glass vials were left to incubate at room temperature without shaking, and the biofilm formation was observed over the specified period. At every sampling interval, the contents in the glass vials were discarded and thoroughly rinsed with distilled water to remove poorly adhered cells. Next, the crystal violet (200 µL, 0.2%) dye was aliquoted into the glass vials to stain the contents. Finally, the glass vials were rinsed thoroughly with $ddH_2O$ to remove excess crystal violet stain. Subsequently, the crystal violet stain was eluted with 95% ethanol (Systerm, Malaysia)), and the absorbance level was measured using the UV Spectrophotometer (Eppendorf, Germany) at 550 nm for each respective sample. The biofilm formation of a probiotic mixture, constituent single strain probiotics, and *A. hydrophila* was recorded and compared.

### Minimum Inhibitory Concentration (MIC) of the Potential Probiotic Mixture against *Aeromonas hydrophila*

The probiotic mixture of *B. amyloliquefaciens* L9, *L. fusiformis* A2, and *E. hirae* LAB3 was further analyzed for its inhibitory potential against *A. hydrophila*. Minimum inhibitory concentration is done to determine the lowest concentration of the potential probiotic mixture that can inhibit the growth of pathogenic *A. hydrophila* in a liquid medium (Andrews, 2001). Before the assay, selected single strain probiotics and *A. hydrophila* were cultured overnight in the TSB medium. The next day, the potential probiotics were combined to produce a probiotic mixture and allowed to incubate for 20 min. Absorbance levels of both probiotic mixture and pathogen were measured using the UV Spectrophotometer (Eppendorf, Germany) at 550 nm. The pathogen was adjusted to the concentration of $10^6$ CFU $mL^{-1}$. Next, each concentration of the potential probiotics ($10^1$ CFU $mL^{-1}$ to $10^9$ CFU $mL^{-1}$) was inoculated and cultured individually with *A. hydrophila* at $10^6$ CFU $mL^{-1}$ in Falcon tubes. The tubes were allowed to incubate overnight on an orbital incubator shaker (BioSan Laboratories Inc., Latvia) at 30 °C. The following day, 1 mL from each tube containing different concentrations of the probiotic mixture co-cultured with the pathogen was transferred into 1.5 mL Eppendorf tubes for serial dilution to ease the counting of colonies. The serially diluted cultures (100 uL) were plated on *Aeromonas* Isolation Medium Base (HiMedia, India). The plates were incubated overnight at 30 °C, and afterward, the colonies of *A. hydrophila* were counted as colony-forming units per mL (CFU $mL^{-1}$) using the following formula:

$$\text{CFU mL}^{-1} = \frac{\text{(Number of colonies) x (Dilution factor)}}{\text{Volume of culture plate (mL)}}$$

### Co-culture Assay of the Potential Probiotic Mixture with *Aeromonas hydrophila*

The co-culture assay is a liquid medium used to observe and quantify the interaction between the potential probiotic mixture and pathogen over time (Vaseeharan & Ramasamy, 2003). As determined from the minimum inhibitory concentration assay, the concentration of the potential probiotic mixture used was $10^7$ CFU $mL^{-1}$, whereas the concentration of pathogen, *A. hydrophila*, was 106 CFU $mL^{-1}$. Samples were taken at 0-, 6-, 12-, 24-, 48-, and 72-h. At each sampling interval, 100 µL of the co-culture treatment was serially diluted for the ease of counting the colonies and proceeded to plate on *Aeromonas* Isolation Medium Base (HiMedia, India). Then, suitable dilution was aliquoted onto the medium, and colonies of *A. hydrophila* were counted as CFU $mL^{-1}$ using the formula below:

$$\text{CFU mL}^{-1} = \frac{\text{(Number of colonies) x (Dilution factor)}}{\text{Volume of culture plate (mL)}}$$

### Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics 20 software. All data collected were analyzed using a one-way analysis of variance (ANOVA). In addition, Tukey's test was applied for pairwise comparison of the means. Data

were expressed as mean ± standard error of the mean (SEM) at a significant level of $P < 0.05$.

## RESULTS

### Antagonistic Screening of Potential Probiotics and Mixture

All three potential probiotics had an inhibitory effect against *A. hydrophila*, as indicated by the clear zone around the well (Figure 1). The inhibitory zone recorded by *B. amyloliquefaciens* L9 was 14.5 ± 0.3 mm (Table 2), whereas *L. fusiformis* A2 has the highest inhibitory zone size with an average of 15.5 ± 0 mm. Contrarily, *E. hirae* LAB3 only showed the presence of inhibitory activity, but the inhibition zone could not be quantified. Therefore, a well diffusion assay was conducted using the mixture to ensure that the antagonistic activity of the probiotic mixture against *A. hydrophila* was conserved. Zones of inhibition were measured and recorded accordingly in Table 2. The probiotic mixture (L9 + LAB3 + A2) inhibited the growth of *A. hydrophila* with an inhibition zone size of (15 ± 0) mm.

Table 2

*Antagonistic screening of potential* probiotics *against* Aeromonas hydrophila

| Potential probiotic | Strain | Diameter of inhibition zone (mm) ± SEM |
|---|---|---|
| *Bacillus amyloliquefaciens* | L9 | 14.5 ± 0.3 |
| *Enterococcus hirae* | LAB3 | + |
| *Lysinibacillus fusiformis* | A2 | 15.5 ± 0 |
| Probiotic mixture | L9 + LAB3 + A2 | 15 ± 0 |

*Note*. Size of inhibition zone ± SEM, n = 3; + = The presence of inhibitory zone but not measurable

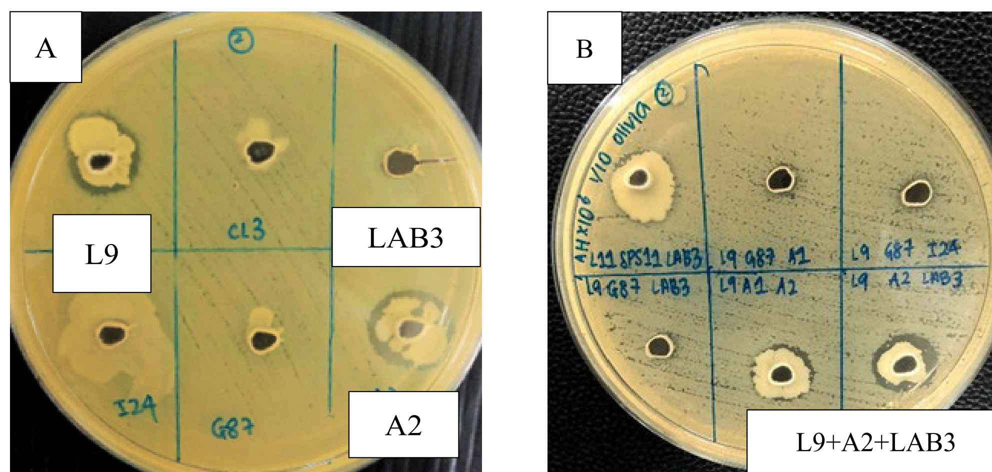


*Figure 1*. Inhibition zones of potential probiotic strains against *Aeromonas hydrophila* at a concentration of $10^6$ CFU $mL^{-1}$ were produced in *in vitro* well diffusion assay. Clear zones indicate the inhibitory activity by the respective probiotic mixture (A) = *Bacillus amyloliquefaciens* (L9), *Enterococcus hirae* (LAB3), *Lysinibacillus fusiformis* (A2); (B) = L9 + LAB3 + A2 (Probiotic mixture)

## Compatibility Assay of Selected Probiotics

The selected probiotics, consisting of *B. amyloliquefaciens* L9, *L. fusiformis* A2, and *E. hirae* LAB3, were tested for their compatibility. All probiotics showed no presence of inhibition when tested against each other. It indicates that the functionality of each probiotic will not be affected when combined into a mixture.

## *In vitro* Hemolysis Assessment

The probiotic mixture showed a lack of hemolysis activity in the area surrounding the bacterial colony and was classified as γ- hemolysis (Figure 2). In contrast, *A. hydrophila* exhibited a clear zone of hemolysis representing the complete breakdown of the hemoglobin of the red blood cells in the vicinity of the bacterial colony and was classified as β-hemolysis.

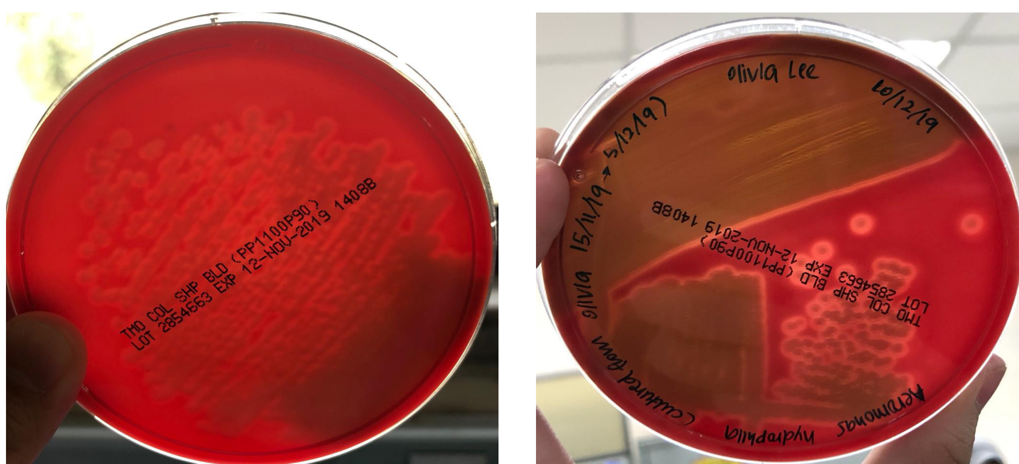


*Figure 2*. Potential probiotic mixture (containing *Bacillus amyloliquefaciens* L9, *Lysinibacillus fusiformis* A2, and *Enterococcus hirae* LAB3) (Left) and *Aeromonas hydrophila* (Right) streaked on the blood agar

## Biofilm Production Assay

Biofilm assay determines the probiotic mixture's ability to produce biofilm to confer protection against *A. hydrophila*. During 6-, 12-, 24-, 48-, and 72-h, the absorbance of the stained-biofilms produced by *A. hydrophila*, probiotic mixture (*B. amyloliquefaciens* L9, *L. fusiformis* A2, and *E. hirae* LAB3), and its single probiotics were shown in Figure 3.

The biofilm formation of the probiotic mixture showed an increasing trend from 6- to 72-h. The absorbance reading for the probiotic mixture was significantly higher ($P > 0.05$) (2.441 ± 0.346) than the pathogen *A. hydrophila* (0.578 ± 0.124) at 24-h interval. Moreover, the biofilm production of single-strain probiotics showed no significant results. Hence, the probiotic mixture harbors a synergistic effect to produce a higher amount of biofilm.

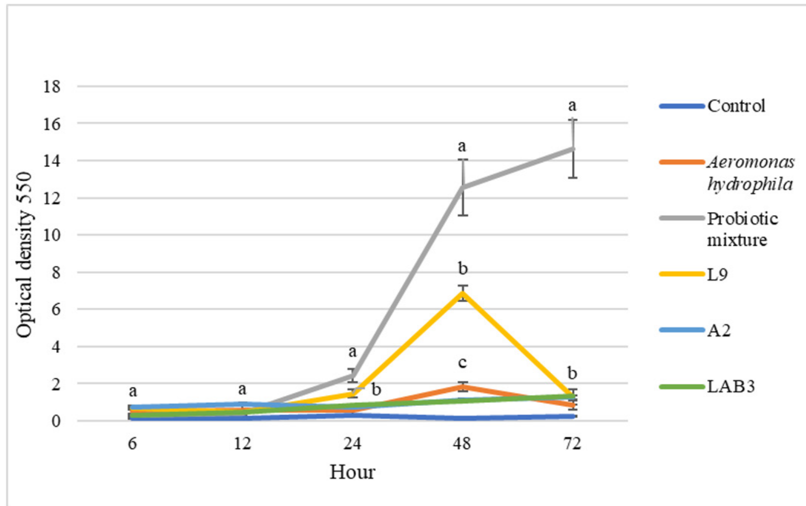


*Figure 3*. The biofilm production by *Aeromonas hydrophila*, probiotic mixture (L9 + A2 + LAB3), and single-strains probiotics (L9, A2, and LAB3) during 6-, 12-, 24-, 48-, and 72-h. Different alphabets indicate significant differences among treatments ($P < 0.05$)

*Note*. L9 = *Bacillus amyloliquefaciens*; A2 = *Lysinibacillus fusiformis*; LAB3 = *Enterococcus hirae*

## Minimum Inhibitory Concentration of the Potential Probiotic Mixture Against *Aeromonas hydrophila*

The assay was carried out to determine the lowest concentration of probiotic mixture that could inhibit the growth of *A. hydrophila* in liquid mode when they were cultured together in TSB. The *A. hydrophila* count reduced significantly ($P < 0.05$) when treated with $10^7$, $10^8$, and $10^9$ CFU $mL^{-1}$ of the probiotic mixture (Figure 4). Nevertheless, the probiotic mixture at concentrations ranging from $10^1$ to $10^6$ CFU $mL^{-1}$ showed no significant effect ($P > 0.05$) in inhibiting the growth of *A. hydrophila*. Hence, the lowest concentration of probiotic mixture required to inhibit the growth of *A. hydrophila* was $10^7$ CFU $mL^{-1}$.

## Co-Culture Assay of the Potential Probiotic Mixture with *Aeromonas hydrophila*

Based on the minimum inhibitory concentration assay, the lowest concentration of probiotic mixture that significantly reduced ($P < 0.05$) the growth of *A. hydrophila* was $10^7$ CFU $mL^{-1}$. The MSP concentration at $10^7$ CFU$mL^{-1}$ was further used to analyze the interaction between the probiotic mixture and *A. hydrophila* during 0-, 6-, 12-, 24-, 48-, and 72-h (Figure 5). The co-culture treatment (T2) ($10^7$ CFU $mL^{-1}$ probiotic mixture + $10^6$ CFU $mL^{-1}$ *A. hydrophila*) demonstrated a gradual significant decrement ($P < 0.05$) in pathogen count at all time intervals. The most significant reduction ($P > 0.05$) observed was at 72-h, where the probiotic mixture reduced the *Aeromonas* count from $8.91 \pm 0.02$ CFU $mL^{-1}$ to $5.781 \pm 0.01$ CFU $mL^{-1}$.

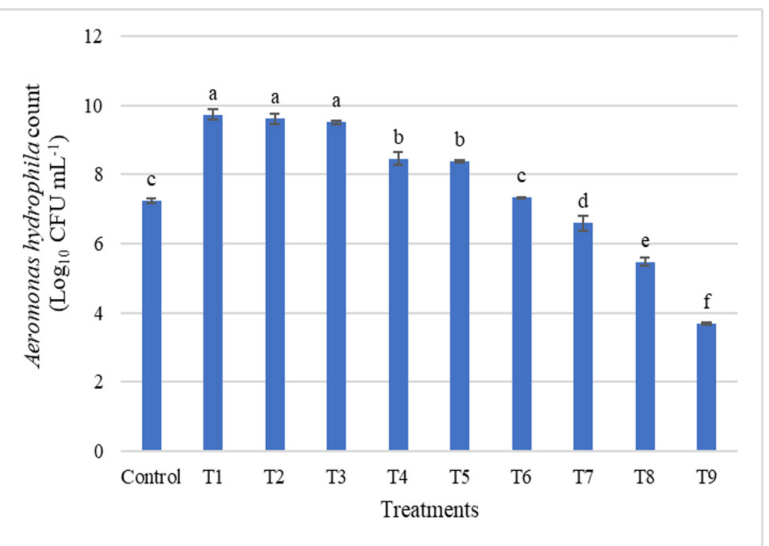


*Figure 4*. The inhibition of *Aeromonas hydrophila* by the probiotic mixture (L9 + A2 + LAB3) at different concentrations after 24 h incubation at 30 °C. Different alphabets indicate significant differences among treatments ($P < 0.05$)

*Note.*
Control = *Aeromonas hydrophila* $10^6$ CFU $mL^{-1}$
T1 = Probiotic mixture $10^1$ CFU $mL^{-1}$ + *Aeromonas hydrophila* $10^6$ CFU $mL^{-1}$
T2 = Probiotic mixture $10^2$ CFU $mL^{-1}$ + *Aeromonas hydrophila* $10^6$ CFU $mL^{-1}$
T3 = Probiotic mixture $10^3$ CFU $mL^{-1}$ + *Aeromonas hydrophila* $10^6$ CFU $mL^{-1}$
T4 = Probiotic mixture $10^4$ CFU $mL^{-1}$ + *Aeromonas hydrophila* $10^6$ CFU $mL^{-1}$
T5 = Probiotic mixture $10^5$ CFU $mL^{-1}$ + *Aeromonas hydrophila* $10^6$ CFU $mL^{-1}$
T6 = Probiotic mixture $10^6$ CFU $mL^{-1}$ + *Aeromonas hydrophila* $10^6$ CFU $mL^{-1}$
T7 = Probiotic mixture $10^7$ CFU $mL^{-1}$ + *Aeromonas hydrophila* $10^6$ CFU $mL^{-1}$
T8 = Probiotic mixture $10^8$ CFU $mL^{-1}$ + *Aeromonas hydrophila* $10^6$ CFU $mL^{-1}$
T9 = Probiotic mixture $10^9$ CFU $mL^{-1}$ + *Aeromonas hydrophila* $10^6$ CFU $mL^{-1}$

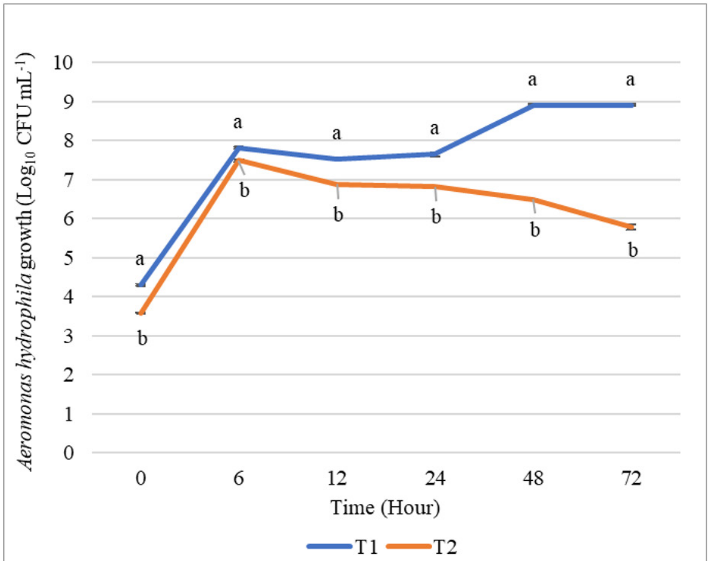


*Figure 5*. The growth of *Aeromonas hydrophila* ($10^6$ CFU $mL^{-1}$) co-cultured with the probiotic mixture (L9 + A2 + LAB3) ($10^7$ CFU $mL^{-1}$) during 0-, 6-, 12-, 24-, 48-, and 72-h. Different alphabets indicate significant differences among treatments

*Note.*
T1 = *Aeromonas hydrophila* $10^6$ CFU $mL^{-1}$
T2 = Probiotic mixture $10^7$ CFU $mL^{-1}$ + *Aeromonas hydrophila* $10^6$ CFU $mL^{-1}$

## DISCUSSION

Aquaculture has been increasingly viewed as an important sector for food security with the growing global human population. The industry has drastically developed due to the intensification of cultivation methods. However, disease outbreaks are the most significant constraint that causes damaging effects on the economic development of the aquaculture sector worldwide (Hai, 2015). Moreover, antimicrobials have put pressure on developing a more sustainable alternative. Therefore, the screening of beneficial probiotics through *in vivo* and *in vitro* assessments are necessary for supplementing aquatic animals to obtain favorable results (Van Doan et al., 2021). For instance, in this study, a potential probiotic mixture (L9 + LAB3 + A2), which showed no hemolytic action and showed high biofilm formation, significantly inhibited the growth of *A. hydrophila* when tested *in vitro*.

The efficiency of antagonistic activity is one of the modes of action of probiotics (Yi et al., 2019). Thus, it is a critical prerequisite when screening for potential probiotics. Furthermore, the higher growth rate of probiotics compared to pathogenic microbes causes the exclusion of pathogenic microbes due to competition for adhesion sites (Kuebutornye et al., 2020). Thus, the present study indicated that the inhibitory activities of the potential probiotics against *A. hydrophila* suggest them as promising candidates for a probiotic mixture.

The formulation of multi-species probiotics is vital as they confer beneficial synergistic effects to the host (Puvanasundram et al., 2021). In this study, the antagonism between selected single strain probiotics and *A. hydrophila* reflects the antagonistic activities of the multi-species probiotic mixture. The compatibility between *B. amyloliquefaciens* L9, *L. fusiformis* A2, and *E. hirae* LAB3 indicated that these probiotic strains could be used in a probiotic mixture without affecting each other's functionality in the host (Toscano et al., 2014).

A safety assessment test is important for the evaluation of probiotics. The absence of hemolytic activity by the potential probiotics suggested that the probiotic mixture is non-virulent and lacks hemolysin (Yasmin et al., 2020). Hemolysis is the breakdown of the membrane of red blood cells by a bacterial protein known as hemolysin, which catalyzes the release of hemoglobin from the red blood cells. Many researchers reported that probiotics should not show any hemolytic activity (Kaktcham et al., 2018; Nayak & Mukherjee, 2011).

Biofilms play a major role in bacterial proliferation and persistence by assisting in the tolerance to external impacts, including antimicrobials (Flemming et al., 2016). The mechanism of biofilm production by probiotic strains is to outcompete pathogenic bacteria for the source of nutrients and habitat colonization (Bhandary et al., 2021). The current results demonstrated that the potential probiotic mixture, when supplemented with a host, may provide functionality in competing against *A. hydrophila* in the gut, thus protecting the

host. The present findings also suggested that the potential probiotics synergistically complement each other when combined into a probiotic mixture and could be more effective in producing biofilm than its constituent single-strain probiotics.

The current study showed that the co-culture between the probiotic mixture ($10^7$ CFU $mL^{-1}$) and *A. hydrophila* caused a reduction in bacterial count on the *Aeromonas* selective media. Furthermore, a higher concentration of probiotic strain *L. fusiformis* SPS11 ($10^8$ CFU $mL^{-1}$) reduced the growth of *Vibrio parahaemolyticus* after 6 hours of incubation (Zabidi, Rosland, et al., 2021). The results from the co-culture assay suggested that the potential probiotic mixture may confer protection against *A. hydrophila* infection when supplemented with a host. Similarly, *Oreochromis mossambicus* fed with a diet containing *Bacillus licheniformis* at $10^7$ cfu $g^{-1}$ improved the health status and resistance of the host against *A. hydrophila,* with the highest value of relative percentage of survival (RPS) of 71.2% (Gobi et al., 2018).

However, other than competitive exclusion as a probable mechanism of action of a probiotic mixture, the production of inhibitory substances could be the facilitator in antagonizing pathogenic *A. hydrophila*. Generally, microbial populations may release chemical substances with bactericidal or bacteriostatic properties. These substances halt the proliferation of pathogenic bacteria (El-Saadony et al., 2021).

*Bacillus amyloliquefaciens* L9 and *L. fusiformis* A2 are Gram-positive bacteria from the genus of *Bacillus* known as spore-forming bacteria resistant to aggressive physical and chemical conditions (Soltani et al., 2019). *Bacillus* species can produce many antimicrobial metabolites and bioactive peptides, such as bacteriocins, bacteriocin-like substances, and lipopeptides (Stein, 2005). Some major probiotics consist of LAB (Alonso et al., 2019). The genus from LAB includes *Enterococcus*, which are Gram-positive microorganisms. Furthermore, LAB is known to produce compounds, such as bacteriocins, that inhibit the growth of other microorganisms (Vandenbergh, 1993).

Conclusively, probiotics have many mechanisms of action yet to be elucidated. However, in this study, it could be speculated that competitive exclusion and production of inhibitory substances are the main mechanisms. However, future *in vivo* studies should confirm probiotics' potential in protecting the host from disease caused by *Aeromonas*. It is because *in vitro* testing is insufficient in selecting probiotics for use in aquaculture, as some studies indicated that positive *in vitro* results may not be reflected in the *in vivo* trials towards the host (Kesarcodi-Watson et al., 2008).

## CONCLUSION

The *in vitro* assessment of the potential probiotic mixture (consisting of *B. amyloliquefaciens* L9, *L. fusiformis* A2, and *E. hirae* LAB3) had shown promising prospects in substituting antibiotics as an eco-friendlier approach. This study serves as a platform for future research into other

properties and mechanisms of action of the probiotic mixture. Further *in vivo* challenge with *A. hydrophila* on freshwater aquatic animals treated with the probiotic mixture is necessary. Hence, the *in vivo* test shall determine if the probiotic mixture has the potential to protect aquatic animals against a bacterial pathogen.

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# Isolation, Characterization, and Optimization of Keratinase from *Bacillus cereus* BRAW_KM

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## ABSTRACT

Indonesia possesses tremendous marine resources. Therefore, their marine products are appropriate for exploration. In the prior study, bacteria generating keratinase enzyme have isolated from local fish market trash. The keratinase may hydrolyze keratin on the skin. Surrounding parameters, such as temperature, pH, and incubation duration, are the factors affecting the activity of the enzyme. This study aims to isolate and characterize keratinase, and optimize its production. The enzyme from *Bacillus cereus* BRAW_KM was the main material utilized in this research. First, the keratinolytic bacterium was isolated and investigated the properties of keratinase using native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE. Then, the ideal conditions of keratinase synthesis were adjusted by temperature, pH, and incubation time on enzyme activity. Of 10 isolations discovered, one isolate shows the potential as a keratinolytic bacterium, which tends to behave like *Bacillus* sp. The molecular weights of keratinase were 130 kDa and 95 kDa. The optimum keratinase enzyme activity from *B. cereus* BRAW_KM was at 29 °C, pH 9, and 90 minutes of incubation.

*Keywords*: *Bacillus cereus*, characterization, isolation, keratinase enzyme, optimization

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## INTRODUCTION

Indonesia is the world's biggest archipelagic state, with 54,716 kilometers of coastline and 17,508 islands, and the world's fourth most populated country, with 247.5 million people (Food and Agriculture Organization of the United Nations [FAO], 2019). Indonesia, behind China, is the world's second-largest producer of fisheries and

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aquaculture. Indonesia's fish production has risen steadily during the last 50 years. Fish supply climbed to 10.7 million tons in 2014, up from 0.8 million tons in 1960 (Tran et al., 2017). The catch is dried, fermented, salted, boiled, or smoked in various proportions, with 46% consumed fresh from the fish market (FAO, 2019).

Wibowo et al. (2017b) identified the bacteria that produce keratinase from fish market waste, which was later described and optimized by Wibowo and Yuliatmo (2020). This enzyme is employed in the hair removal (unhairing) procedure for eco-friendly fish skin tanning. According to Tamersit and Bouhidel (2020), the unhairing procedure results in a highly polluted solution. Dettmer et al. (2013) also explained that the unhairing process leads to the destruction of hides hairs can use the conventional lime-sulfide method, even though this method causes emissions. High biological oxygen demand (BOD), total suspended solids (TSS), and chemical oxygen demand (COD) are the emission that loads in the effluent leather industry. Furthermore, protein degrading chemical materials, such as calcium carbonate ($CaCO_3$) and sodium sulfide ($Na_2S$), are used in leather manufacture. It accounts for about 80-90% of the total pollution in leather making (Dettmer et al., 2012). For environmental concerns and to reduce sodium sulfide usage for the tanning process, the keratinase enzyme can be used, which is important for the tanning process and future technology (Kandasamy et al., 2012).

The employment of enzymes in the tanning process is a great future trend. The keratinolytic enzymes have been discovered by researchers. Most of them are derived from *Bacillus* strains, such as *Bacillus* BPKer and BAKer (Gegeckas et al., 2018), *Bacillus aerius* NSMk2 (Bhari et al., 2019), *Bacillus cereus*, and *Bacillus polymyxa* (Laba & Rodziewicz, 2014); *Bacillus subtillis* is among others (Mousavi et al., 2013). Several parameters can impact enzyme production, including pH, temperature, and incubation time. Condition optimization is an important aspect of enzyme production (Mechri et al., 2017). In this research, the isolation, characterization, and optimization of keratinase from *Bacillus cereus* BRAW_KM is expressed as an innovative solution to support cleaner production in leather tanning factories.

## METHODS

### Isolation of *Bacillus cereus* BRAW_KM

Samples of Buntal fish skin were obtained from a local fish market in Rembang, Center of Java, Indonesia. Nutrient agar (Merck, Germany) was used for inoculation of the sample and incubated for 48 h at 37 °C until colonies appeared. Colonies were selected as representative samples based on morphological and colony color observations. Then, the selected colonies were isolated by transferred to the new NA plates (Wibowo et al., 2017b).

### Identification of *Bacillus cereus* BRAW_KM

**Proteolytic Activity by Skim Milk Agar Identification.** The isolate was then streaked on skim milk agar (Merck, Germany)

(0.8% skim milk, 0.5% sodium chloride [NaCl], 1% meat extract, 1% peptone, 1.5% agar) and incubated for 48 h at 37 °C. After incubation, a clear zone around the bacterial growth was observed (Wibowo et al., 2017a). All chemicals were used in this study purchased from Merck (Germany).

**Scanning Electron Microscope.** Cells bacteria grown in NA medium were harvested after 72 h of incubation and subjected to scanning electron microscopy (SEM) analysis (PerkinElmer, USA). The 0.22 M sucrose (Merck, Germany) in cacodylate buffer (0.1 M, pH 7.2) (Merck, Germany) was used to wash the cell, which was subsequently fixed cacodylate buffer containing 2% (v/v) glutaraldehyde (Merck, Germany) at 4 °C for 2 h. The suspension was centrifuged before being rinsed in cacodylate buffer once more. The samples were fixed in cacodylate buffer containing 1% osmium tetroxide (Merck, Germany) at 4 °C for 2 h, dehydrated by gradation of alcohol (Merck, Germany) concentration, and dried in hexamethyldisilane (Merck, Germany) and mounted on aluminum stubs. The sample is sputter-coated with gold/palladium, then viewed using SEM.

**Morphology Test.** Bacterial identification was performed by observing colony morphology such as texture, shape, size, motility, colony color, zinc (Zn) staining, and Gram staining. At the same time, the biochemical test includes oxidase, catalase, and fermentation of carbohydrates. The results were compared to the standard from Bergey's Manual of Determinative Bacteriology (Bergey & Gibbons, 1974).

**Keratinase Enzyme Production**

**Inoculum Preparation for Enzyme Production.** The following sources were utilized in this research: *B. cereus* BRAW_KM was isolated from the culture of a previous study (Wibowo et al., 2017a). The fermentation medium contained the following ingredients: 0.5 g/L NaCl, 0.3 g/L dipotassium hydrogen phosphate ($K_2HPO_4$), and 0.4 g/L potassium dihydrogen phosphate ($KH_2PO_4$), 1% yeast extract, 0.5% NaCl, 1% peptone, and 100 mL distilled water, 1% ammonium sulfate ($(NH_4)_2SO_4$), 20 mM Tris (hydroxymethyl) aminomethane hydrochloride (pH 8), 12 kDa dialysis sheet, 1 mM ethylenediamine tetraacetic acid (EDTA), 50 mM sodium bicarbonate ($NaHCO_3$), and distilled water. Five (5) mL of preculture medium were inoculated with one dose of pure culture product isolate obtained from agar media and incubated overnight at 120 rpm in a shaker.

**Enzyme Production.** The method developed by Hoq et al. (2005) was used to produce the keratinase enzyme. Approximately 1.5 mL of the isolate was inoculated into a 50 mL liquid medium and incubated overnight at 120 rpm in a shaker. Yellowing is a characteristic of enzyme production. The isolates were separated from the extracellular enzyme by centrifugation for 15 minutes at 4 °C and 1, 400 × *g*. The generated supernatant was a raw enzyme that enzyme activity could be tested. The

enzyme activity of the collected enzyme was determined.

**Enzyme Purification.** At 4 °C, a one-liter culture product of fermentation was centrifuged for 15 minutes at 10,000 × *g*. The pellet was separated from the supernatant that had been formed. The enzyme extract present in the supernatant was able to be concentrated due to the concentration method. The enzyme was refined by precipitating it with saturated ammonium sulfate at a concentration of 60% for many hours. Saturation was accomplished using ammonium sulfate (Tatineni et al., 2008). The ammonium sulfate crystals were gently added while constantly swirling until entirely dissolved. The solution was stored at 4 °C for 24 h before being centrifuged at 10,000 × *g* for 15 minutes at 4 °C. Following centrifugation, the pellets and supernatant are separated. The pellets are collected and considered the purified enzyme, while the supernatant is removed and considered as other nutrients such as saccharides and minerals (Yuliatmo et al., 2017).

**Characterization of Enzyme.** ***Determination of Molecular Weight of Protein by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).*** In addition to ethanol, other SDS-PAGE components include sterile water, acrylamide solution, Tris hydrochloride (Tris-HCl), SDS, deionized water ($dH_2O$), N'-tetramethyl-ethylenediamine (TEMED), ammonium persulfate, glacial acetic acid, Coomassie blue, and 70% methanol. All the buffers used in the tests, including sodium phosphate, glycine, sodium hydroxide (NaOH), and Tris-acetate, were used. Some of the materials used to assess the activity of the enzyme keratinase were keratin azure, Tris HCl (pH 7.5), and a 10% trichloroacetic acid (TCA) solution, among other things. In the case of protein separation, the SDS-PAGE method is used to determine the molecular weight of the proteins being separated. In SDS-PAGE, the essential concept is that proteins are denatured by sodium dodecyl sulfate, followed by molecular weight separation by electrophoresis using a gel, in this case, polyacrylamide, to separate proteins with varying molecular weights, as described above. On SDS-PAGE, the identification and characterization of protein bands were carried out in comparison to bands that had previously been separated using conventional protein separation methods (Laemmli, 1970).

***Protease Activity by Native PAGE.*** Protease activity of the enzyme was performed by Hiol et al. (1999) using clear native PAGE (CN-PAGE). The 10% concentrated CN-PAGE contained 30% acrylic amide solution; 0.8% bisacrylamide; 1.5 M Tris hydrochloride (pH 8.8); 1.0 M Tris hydrochloride (pH 6.8), 0.8% 1,2-Bis(dimethylamino)ethane, 10% ammonium persulfate, 50% glycerin, 0.1% casein, TEFCO clear dry (Japan), running buffer solution (1.5 g Tris (hydroxymethyl) aminomethane, 7.2 g acetohydroxamic acid ($C_2H_5NO_2$), and 500 mL distilled water),

and 70 mm ADVANTEC filter paper (Toyo Roshi Kaisha, Japan).

**Optimization of Enzyme Production.** ***Keratinase Activity.*** Keratin azure (Sigma-Aldrich, USA) was used as a keratin substrate to determine the keratinase activity. The keratinase activity test is based on Wang et al. (2009). An incubation period of 30 minutes at 30 °C with the agitation of 180 rpm was carried out in a shaker incubator with a 500 mL enzyme sample in 5 mg keratin azure solution in 500 mL 50 mM sodium phosphate buffer at 50 mM sodium phosphate buffer. The process was stopped by adding 1 mL of 10% TCA solution to the mixture. Centrifuging the solution at 13,000 × *g* for 5 minutes after it had been maintained cool was the first-rate procedure. It was necessary to measure the absorbance of the azo dye extracted from the supernatant at 595 nm to compare it to the absorbance of the control tube. The control tube was subjected to the identical procedures as the experimental tube, with the exception that the enzyme sample was replaced with sodium phosphate buffer instead of phosphate buffer. One unit (U) keratinase activity was defined as the amount of enzyme causing a 0.01 absorbance increase between the sample and control at 595 nm under the conditions given.

***The Effects of Temperature, pH, and Incubation Time on Keratinase Activity.*** The purified enzyme's keratinase activity was determined using the following buffers (sodium acetate [$CH_3COONa$, pH 4–6], trisodium phosphate [$Na_3PO_4$, pH 7–8], Tris sodium hydroxide [Tris-NaOH] [pH 9-11]) at pH 6, 7, 8, 9, 10, and 11. By incubating processes at a variety of temperatures, including 25 °C and 27 °C, as well as 29 °C, 31 °C, and 33 °C, the optimal temperature was identified. Additionally, the incubation length for keratinase characteristics varied from 30 minutes, 60 minutes, 90 minutes, 120 minutes, and 150 minutes, according to the experiment results (Nayaka et al., 2013).

***Determination of $V_{max}$ and $K_m$.*** The enzyme kinetic parameters Michaelis constant ($K_m$) and maximum rate of reaction ($V_{max}$) were obtained by analyzing keratinase activity at various substrate concentrations (1-10 mg). With the use of the Lineweaver-Burk plot, the values for $K_m$ and $V_{max}$ were determined. Several researchers (Gupta et al., 2015) have suggested that.

## RESULTS AND DISCUSSION

### Isolation of *Bacillus cereus* BRAW_KM

Ten strains of bacteria have been successfully collected. It shows the clear zone on a skim milk agar plate, indicating its ability to degrade casein protein (Figure 1A). In addition, skim milk agar is the best medium for the preliminary screening of keratinase (Balakumar et al., 2013; Selvam et al., 2013).

### Identification of *Bacillus cereus* BRAW_KM

Bacteria morphology is essential for identification. It was occurred by SEM. The observations using a microscope showed that the bacteria form is small

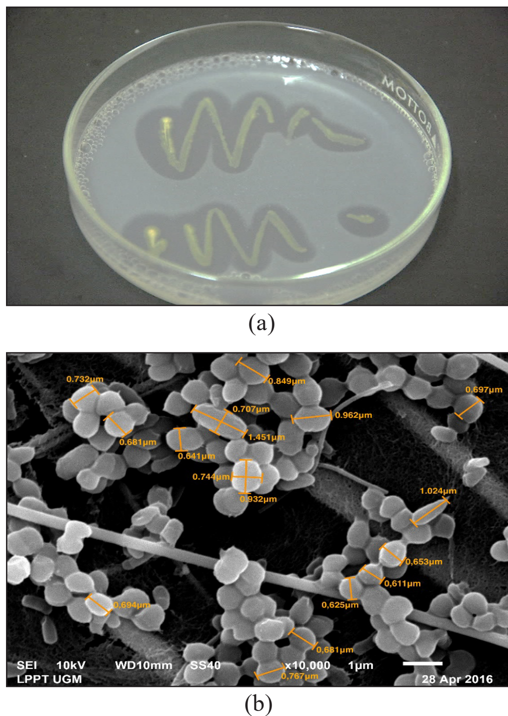


*Figure 1.* (a) Bacteria growth on skim milk agar; (b) Scanning electron microscope of isolating *B. cereus* BRAW_KM

Table 1
*Morphology and biochemical characteristics*

| Characteristics | Results |
|---|---|
| Catalase | + |
| Oxidase | + |
| Deep media | Beaded |
| Slant media | Echinulate |
| Elevation | Effuse |
| Edge | Entire |
| Inner structure | Translucent |
| Colony form | Circular |
| Glucose | + |
| Fructose | + |
| Sucrose | + |
| Lactose | + |
| Motility | - |
| Spore | + |
| Gram staining | + |
| Acid staining (Zn) | - |

*Note.* + = Positive reaction; - = Negative reaction

rod shape, almost round (Figure 1B). Morphological, physiological, and biochemical characteristics are represented in Table 1. The characteristics assay results showed that these bacteria were classified as *Bacillus* sp. (Wibowo et al., 2017a).

## Characterization of Keratinase

**Molecular Weight Determination Using SDS-PAGE.** Electrophoresis is widely used for protein characterization, including the measurement of molecular protein weight. By estimating the molecular protein weight of the enzyme after it has been exposed to SDS-PAGE, the molecular weight of the enzyme may be determined and matching it with the band in standard protein. For example, molecular weights of enzymes from *Bacillus cereus* strain BRAW_KM are between 130 kDa and 95 kDa. It is in line with Mazotto, Coelho, et al. (2011)'s research that generally, all *Bacillus* spp. genus had keratinases with a molecular weight of 13.8 kDa and 140 kDa. Figure 2 shows the molecular weight of the keratinase of *B. cereus* BRAW_KM. Other extracellular keratinases *Bacillus pumilus*, *Bacillus cereus*, and *Bacillus subtilis* KS-1 had molecules weight 65 kDa, 45 kDa, and 25.4 kDa, respectively (Kumar et al., 2008; Mazotto, de Melo, et al., 2011).

**Native PAGE.** The native PAGE technique was utilized to detect the protein bands and the protease activity of the protein bands, which in this instance was determined to be a specific bacterial strain. According to Sattayasai (2012), enzymatic activity may stain a wide range of proteins in gels.

Wilson and Walker (2010) further stated that the native PAGE procedure does not denature the sample since it has the potential to create bonds with the protein's secondary structure, which would otherwise cause the sample to be destroyed. Native PAGE examination of the enzyme extract from *B. cereus* BRAW_KM using casein substrate (Figure 2) revealed that the enzyme extract could hydrolyze the protein. The protein band of *B. cereus* BRAW_KM could be seen clearly in the image. As a result, the enzyme BRAW_KM from *B. cereus* is capable of degrading protein.

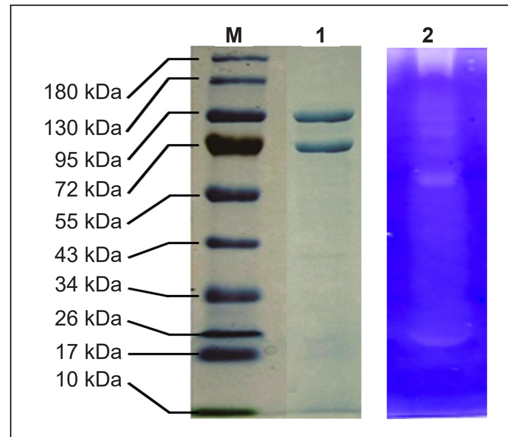


*Figure 2*. Characteristics of keratinase from *B. cereus* BRAW_KM on SDS-PAGE (1) and native PAGE (2), respectively
*Note*. M = Molecule weight standard

## Optimization of Keratinase Production

**The Influence of Temperature on Activity of Keratinase.** Figure 3 represents the effect of temperature on enzyme activity. The best condition for keratinase activity was found by incubating the samples at temperatures ranging from 25 °C to 33 °C. *Bacillus cereus* strain BRAW_KM enzyme has an optimal temperature of 29 °C (6.34 ± 0.03 U/mg), and as the temperature increases, the enzyme's activity drops. It is substantially identical to the findings obtained by Balakumar et al. (2013). *Bacillus subtilis* was inoculated into the medium to maximize keratinase production at different temperatures, and they reported that an increase in production was seen at 30 °C.

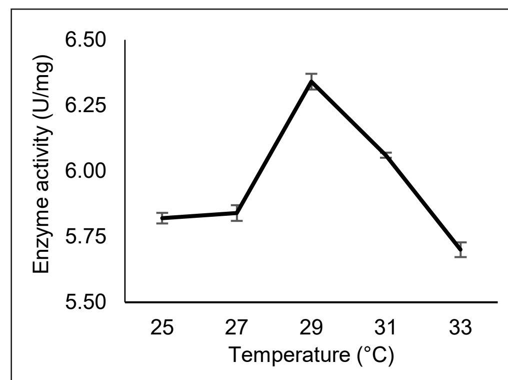


*Figure 3*. The influence of temperature on the activity of keratinase

**The Influence of pH on Activity of Keratinase.** The enzyme from *B. cereus* BRAW_KM activities is optimal at pH 8, with the highest activity at 7.13 ± 0.03 U/mg (Figure 4). It is similar to alkaline protease

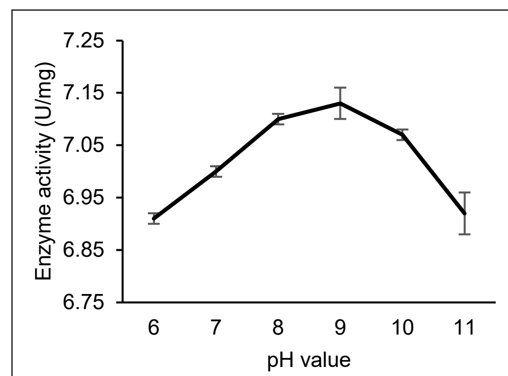


*Figure 4*. The influence of pH value on the activity of keratinase

from APR-4 *Bacillus* sp., which has the maximum activity at pH 9 (Kumar et al., 2008), and to keratinase from *Bacillus* sp., which has the highest activity at pH around 7 and 8 (Selvam et al., 2013). Keratinase produced by these bacterial strains may be classified as an alkaline protease because the enzyme's maximum activity occurs at alkaline pH levels. Lin et al. (1996) explained that high pH treatment does not reduce keratinase activity, but low pH does.

**The Influence of Incubation Time on Activity of Keratinase.** The enzyme activity of *B. cereus* BRAW_KM increases until 90 minutes (6.17 ± 0.02 U/mg), then its activity decrease by 9% after 90 minutes (Figure 5). The optimal incubation period of keratinase from *B. cereus* BRAW_KM almost the same results have been reported by Gupta et al. (2015), who found that keratinase from *B. subtilis* stabilizes up to 90 minutes and drops by 11% after 120 minutes. Other results were seen between the *B. subtilis* proteolytic enzyme BLBc11 (Dettmer et al., 2012) and a commercially available keratinase, with the former demonstrating steady activity for 120 minutes and the latter demonstrating variable activity (Dettmer et al., 2011). According to Gessesse et al. (2003), the enzyme from *Bacillus pseudofirmus* sp. became inactive after 20 minutes of incubation. Apart from that, Ogino et al. (2008) identified proteolytic enzymes that become inactive after just ten minutes of incubation.

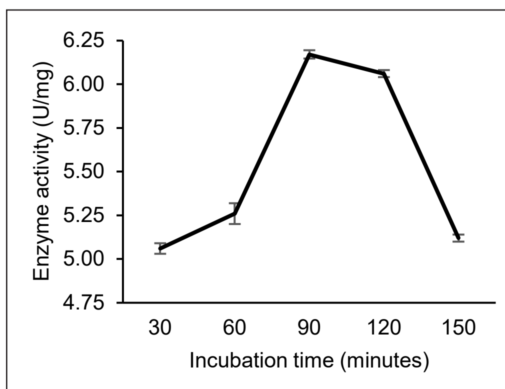


*Figure 5*. The influence of time incubation on the activity of keratinase

## Kinetics of Keratinase

The Michaelis-Menten plot was used to plot Lineweaver-Burk plots against substrates at varying concentrations, and the results were analyzed (Figure 6). According to the

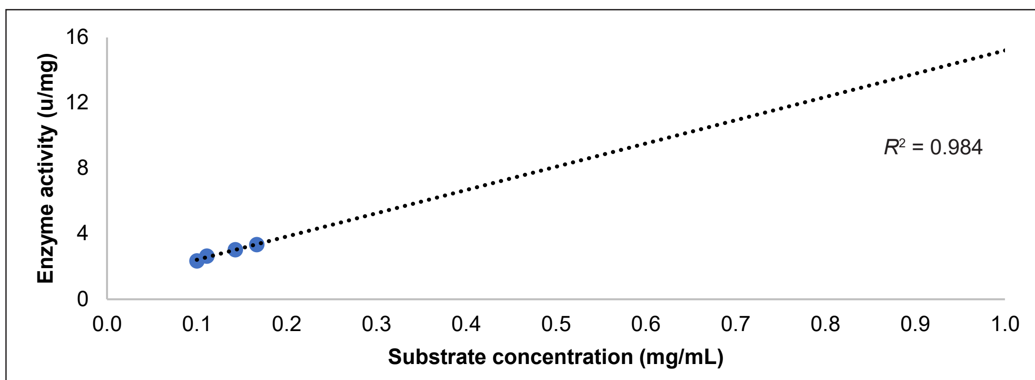


*Figure 6*. Graph of keratinase enzyme kinetics (Lineweaver - Burk plot)

study's results, the Michaelis constant ($K_m$) of keratinase from *B. cereus* BRAW_KM was found to be 13.98 mg/mL, and the maximum rate of reaction ($V_{max}$) was determined to be 1.01 mg/mL/min. The Michaelis-Menten equation was used to estimate the enzyme's reaction at various substrate doses to study enzyme kinetics. Keratinase generated by *Pseudomonas aeruginosa* KS-1 has a higher $K_m$ of 1.66 mg/mL and a higher $V_{max}$ of 3.1 mg/mL/min than previously reported (Sharma & Gupta, 2010). Purified keratinase from *Bacillus thuringiensis* has a greater $K_m$ (5.97 mg/mL) than other keratinases (Sivakumar et al., 2012). The calculated $K_m$ and $V_{max}$ values for keratin obtained from feathers were 6.6 mg/mL and 5.0 mg/mL/min, respectively, for keratin derived from feathers (Gupta et al., 2015).

## CONCLUSION

The bacteria isolation resulted in keratinolytic bacteria, *Bacillus cereus* BRAW_KM. The SDS-PAGE and native PAGE investigated enzymatic characterization. It resulted in the molecular weights of keratinase being 130 kDa and 95 kDa. In addition, temperature, pH, and incubation period on enzyme activity were shown to be the most effective factors in determining the optimal conditions for keratinase synthesis. The best conditions were 29 °C, pH 9, and 90 minutes of incubation.

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# Optimizing Silicon Application to Improve Growth, Grain Yield, and Nutrient Uptake of *indica* Rice (*Oryza sativa* cv. Bw 367)

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## ABSTRACT

The rice plant accumulates silicon (Si) in greater quantity, which varies among the rice genotypes. This study was conducted to determine the optimum fertilization rate and its effect on growth, yield, yield attributes, and soil nutrient uptake. Six different silicon dioxide ($SiO_2$) rates, including 0, 50, 75, 100, 125, and 150 kg $SiO_2$/ha, were applied initially. The optimum rate of $SiO_2$ was obtained by statistical analysis, utilizing the analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) to separate the means. The results showed that shoot dry weight and plant height were significantly affected by Si fertilization. The highest Si tissue concentration of 395.27 µg/100 mg was recorded in 100 kg $SiO_2$/ha treated plants, and their potassium, phosphorous, silicon, and magnesium uptakes were increased by 2, 1.3, 11 and 1.8 folds, respectively. Further, in yield attributes, 32 and 52% increments and a 30% decrease were observed in the total number of grains, filled grains, and unfilled grains per panicle, respectively, and were not significantly different from those observed in 125 kg $SiO_2$/ha rate. The highest grain yield of 104.6 g/pot was obtained with 100 kg $SiO_2$/ha level of Si fertilizer, and it was statistically at par with the yields obtained with 125 kg $SiO_2$/ha. The quadratic function found the rate of Si fertilizer for optimum grain yield (100.5 g/pot) as 115 kg $SiO_2$/ha;

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thus, it could be concluded that *indica* rice genotypes need to be fertilized with 115 kg $SiO_2$/ha for optimum yield for higher growth and nutrient uptake.

*Keywords*: Plant nutrients, silicon accumulation, silicon requirement, yield attributes

## INTRODUCTION

Rice is the primary source of daily calories and a staple food for nearly three billion of the world population, with Asian countries being the greatest consumers. A yield increase of around 50% in major food crops, including rice, has been projected to support the anticipated population by 2050 (Godfray et al., 2010). Therefore, all the rice-producing countries need to be lined up to increase production. Silicon fertilization has been a common practice among the various approaches to increase rice yield. Silicon as a nutrient has multifaceted benefits. Silicon nutrition in the plant has elucidated the beneficial effects of Si on growth and yield improvements in rice plant and its proactive role in mitigating the wide range of abiotic stresses, including drought, salinity, heavy metal toxicities, and biotic stresses, such as pest and disease infestations (Ma & Yamaji, 2006). Silicon enhances the rice plant growth determinants: plant height and shoot weight (Mahendran et al., 2021) and yield parameters, including total grains/panicle, filled grains/panicle, and 1,000 grains weight (Cuong et al., 2017). Moreover, using Si is a high-quality element to improve the productivity in rice lands toward ecologically green agriculture (Liang et al., 2006). Despite the abundance of Si in soil, naturally, the plant-available form of Si in heavily weathered soils in tropical and sub-tropical regions is particularly poor due to fixation with other compounds (Raven, 2003). Furthermore, in paddy lands, plant-available Si greatly decreases with repeated cultivation of high-yielding rice genotypes as a monocrop (Ning et al., 2017) and inadequate Si uptake decreases the rice yield and quality (Jinger et al., 2017). Approximately 20 kg $SiO_2/hm^2$ is taken up by each 100 kg of brown rice from the soil at each harvest (Song et al., 2016), emphasizing the need for exogenous Si application to increase the readily available Si in the soil and for sustainable rice production.

The beneficial effects of Si application on paddy production are related to the accumulated Si in the epidermal tissues. Plants must accumulate a higher amount of Si to obtain its benefits. Commonly, Si deposition occurs in leaves, roots, sheaths, and hulls of the cell walls, making the rice plant more resistant to pest and disease attack, unfavorable abiotic factors, and making the plant stem stronger to resist lodging (Ma & Yamaji, 2006). There is a considerable variation in Si absorption and accumulation in plants depending on the species and the genotypes in the same species. Rice is a heavy Si accumulator which shows wide genotypic variation in tissue Si content (Kim et al., 2012; Ma et al., 2006). Previous studies showed that subspecies *indica*-type rice genotypes significantly vary in the accumulation of Si

(Rupasinghe et al., 2021; Swain et al., 2016). Furthermore, Si concentration in shoots significantly differs between the *indica* and *japonica* genotypes (Gaur et al., 2020; Mitani & Ma, 2005). The Si requirement varied with the yield potential of the rice variety (Gill et al., 2007). Rice genotype Bw 367 is high yielding Si responsive having 105 days maturity period, and a popular short grain *indica* rice genotype grown in Sri Lanka.

Although much research has focused on Si nutrition in *japonica* rice, Si nutrition in *indica* rice genotypes has been sparsely studied. Particularly, data on plant growth performances, including physiological changes, nutrient uptake, and yield performance with the application of Si to *indica* rice genotypes, are inadequate. Therefore, the following experiment was carried out to identify the optimum Si requirement for the maximum yield and to observe the effects of Si on plant nutrient uptake and growth of *indica* rice for sustainable rice production.

## MATERIALS AND METHODS

### Plant Materials and Growing Conditions

A pot experiment was conducted in a plant house at the Universiti Putra Malaysia (UPM) with the test rice genotype Bw 367. It is a Si-responsive rice genotype, which showed the highest growth in nutrient solution fortified with 2mM Si (Sigma Aldrich, Germany) (Rupasinghe et al., 2021). Healthy and uniformed seeds of Bw 367 rice genotype obtained from the Rice Research and Development Institute (RRDI), Sri Lanka, were taken, and 10% hydrogen peroxide ($H_2O_2$) (Sigma Aldrich, Germany) was sprayed with aiming the surface sterilization of seeds. After 10 min, treated seeds were thoroughly rinsed with distilled water for few times. Then seeds were soaked in distilled water for 24 h, followed by incubating in the dark for another 48 h in a Petri dish lined with a moist filter paper to induce germination (Ullah et al., 2017). The pre-germinated seeds were then sown in the pots containing 9 kg of soil. Four seedlings maintained in each pot until harvest, were healthy and uniformly grown. Muriate of potash (MOP, 60% potassium oxide [$K_2O$], Cap Segi Tiga, Belarus), triple super phosphate (TSP, 45% phosphorous pentoxide [$P_2O_5$], ZZ International, China), and urea (46% nitrogen [N], Agrenas, Malaysia) were uniformly applied at the rate of 110, 55, 100 kg/ha, respectively. All experimental units were treated alike with other agronomic practices as well. One of our previous studies identified the optimum Si concentration for the growth of test rice genotype in a hydroponic media as 2 mM (Rupasinghe et al., 2021). Six Si levels as 0, 50, 75, 100, 125, and 150 kg $SiO_2$/ha were formulated as treatments. Silicon was applied as a basal dressing to each pot (Ullah et al., 2017). The experiment was laid out in a randomized complete block design (RCBD) with three replicates per treatment.

**Soil Analysis**

Soil for the study was collected representing the plow depth (0-20 cm) from a paddy land in Sekinchan, Malaysia (3° 6' 19.3" N, 101°28'3.5" E). Then the soil was air dried and sieved using a 2-mm sieve. The initial soil's important physical and chemical characteristics were determined and presented in Table 1. Standard analytical methods were followed to characterize the soil. Electrical conductivity (EC) and soil reaction (pH) were measured using soil: water ratio of 1: 5 and 1: 2.5, respectively, by using an electrical conductivity meter (Mettler Toledo SevenEasy™ Conductivity Meter S30, New Zealand) and pH meter (Model Metrohm 827, USA). Organic carbon content was estimated using the Walkley and Black (1934) procedure. Exchangeable magnesium (Mg) and potassium (K) in soil were extracted using 1M ammonium acetate, pH 7 solution, and measured by atomic absorption spectroscopy (AAS) (AAnalyst 400, PerkinElmer, USA). Available phosphorus was measured using Bray and Kurtz's (1945) method. Available Si (AvSi) in soil was estimated by the method described by Korndörfer et al. (2004).

Table 1
*Important physicochemical characteristics of soils used in this study*

| Parameter | Value |
|---|---|
| Soil pH | 6.16 |
| Electrical conductivity (dS/m) | 0.45 |
| Available phosphorus (mg/kg) | 88 |
| Organic carbon (%) | 1.7 |
| Exchangeable potassium (mg/kg) | 64.1 |
| Cation exchangeable capacity (meq/100 g) | 2.7 |
| Available Si (mg/kg) | 47.4 |
| Texture | Clay |
| Sand (%) | 0.95 |
| Silt (%) | 33.73 |
| Clay (%) | 65.32 |

**Plant Nutrient Analysis**

Straw samples were oven-dried at 80 °C and ground to a fine powder to analyze the nutrient content of phosphorus (P), K, Mg, and Si. P, K, and Mg were extracted using the dry ashing method (Miller, 1998). Briefly, 0.2 g of finely ground plant material was taken into an ashing tube and heated in a muffle furnace for complete ashing under 500 ºC overnight. After cooling the ashing bottles, two drops of deionized water were carefully added to wet the ash. Then 0.5 ml of digestion mixture (25 ml concentrated nitric acid [conc. $HNO_3$] + 25 ml concentrated hydrochloric acid [conc. HCl] made to 100 ml final volume with distilled water) was added into the ashing bottle, followed by evaporating completely on the hot plate under low heat with shaking the mixture. Subsequently, 10 ml of 0.05N HCl was added to the contents and warmed gently to dissolve the residue. Finally, the mixture was vortexed. Phosphorus content in the plant extract was then determined as described in the molybdenum yellow method (Jackson, 1973) using the spectrophotometer (UV-1700 PharmaSpec, Shimadzu Corporation, Japan) at 420 nm wavelength. Magnesium and K in the extracts were measured using the AAS after suitable dilution with distilled

water. Silicon was extracted by adopting the modified auto-clave digestion method (Elliott & Snyder, 1991). A plant sample of 100 mg was digested with 2 ml of 50% $H_2O_2$ and 3 ml of 50% sodium hydroxide (NaOH) in an autoclave under the pressure of 103 kPa for 30 minutes. After extracting Si, the colorimetric molybdenum method was followed to determine the Si concentration by measuring the absorbance at 620 nm using a UV Vis spectrophotometer (UV-1700 PharmaSpec, Shimadzu Corporation, Japan). All the chemicals used were analytical grade with a purity of 99.9% from Sigma Aldrich (Germany).

### Growth and Yield Component

Plant height (cm) was recorded at the harvesting stage (105 days). After separating the grains, the straw was dried at 80 ºC in an oven to a constant weight (48 h) to get the dry weight (g). A number of productive tillers/plants was recorded in each pot. Five panicles of each treatment were randomly selected and counted for total grains/panicle, total filled grains/panicle, total unfilled grains/panicle, and 1,000-grain weight (g) was determined. Finally, pot yield (g) was measured and adjusted to the 12% moisture level.

### Determination of Chlorophyll Content in Plant Tissues

The method explained by Coombs et al. (1985) was applied to determine the chlorophyll content. Briefly, a cork borer was used to take four leaf discs of 4 $cm^2$ from the fully opened third leaf from the top at the panicle initiation stage. Leaf discs were immediately transferred into a glass bottle containing 20 ml of 80% acetone (Sigma Aldrich, Germany) and covered with aluminum foil. Immediately, the bottle was placed in the dark at room temperature for three days until the green color was completely bleached out, confirming the pigments were fully extracted from the leaf discs. Finally, 3.5 ml of the extracted solution was collected in a cuvette to determine the chlorophyll absorbance. The peak absorbance of both chlorophyll *a* and chlorophyll *b* was assessed at two different wavelengths: 664 and 647 nm, respectively, using a spectrophotometer (Cecil, CE1011, 1000 series, United Kingdom). The total amount of chlorophyll was then calculated as follows (Coombs et al., 1985).

Chlorophyll *a* content (mg/$cm^2$ fresh leaf) = 13.19 (A664 – 2.57 (A647) [1]

Chlorophyll *b* content (mg/$cm^2$ fresh leaf) = 22.1 (A647) – 5.26 (A664) [2]

Total chlorophyll content (mg/$cm^2$ fresh leaf) = 3.5 (Chlorophyll *a* + Chlorophyll *b*)/4 [3]

Where A647 and A664 are the solution's absorbance at 647, and 664 nm, respectively, and 13.19, 2.57, 22.1, and 5.26 are the absorbance coefficients, 3.5 is the total volume used in the analysis (ml), and 4 is the area of the whole disc ($cm^2$) used.

### Data Analysis

The data were statistically analyzed by adopting a two-way analysis of variance (ANOVA) using the Statistical Analysis

System (SAS) (version 9.4) (SAS Institute, USA). Duncan's multiple range test (DMRT) at $p < 0.05$ was used to separate means when the effects of treatments were significant. The optimal level of Si fertilizer for maximum grain yield was determined using quadratic regression (Cuong et al., 2017). Finally, Pearson's correlation study was used to find the relationship between Si uptake and selected parameters.

## RESULTS

### Changes in Plant Available Silicon in Soil with Silicon Fertilization

Based on the critical level of Si in the soil, about 40 mg/kg (Nagula et al., 2015), the investigated soil possesses Si concentration of less than the critical level for plant absorption (Table 1). As expected, higher rates (100–150 kg $SiO_2$ /ha) of Si fertilizer have significantly released a higher content of AvSi (Figure 1). At the harvesting stage, this value in soil increased with increasing Si rates, and 100–150 kg $SiO_2$/ha Si rates provided the AvSi content higher than the critical level for rice. However, there was no significant difference in this value for the rates greater than 100 kg $SiO_2$ /ha.

### Effect of Different Rates of Silicon on Growth Parameters

The Si fertilization significantly increased both plant height and the shoot dry weight at the harvesting stage ($p < 0.05$). Among the Si fertilization rates, 100 kg $SiO_2$/ha soil fertilization rate resulted in the tallest plant at 127 cm. The control pot without Si had the shortest plant height of 113.3 cm. However, the plants fertilized with higher Si rates of 125 and 150 kg $SiO_2$/ha were shorter in height than 100 kg $SiO_2$/ha (Figure 2a). Furthermore, the plant achieved its highest relative dry matter accumulation of 52% when Si was fertilized at 100 kg $SiO_2$/ha, followed by 125 kg $SiO_2$/ha (Figure 2b).

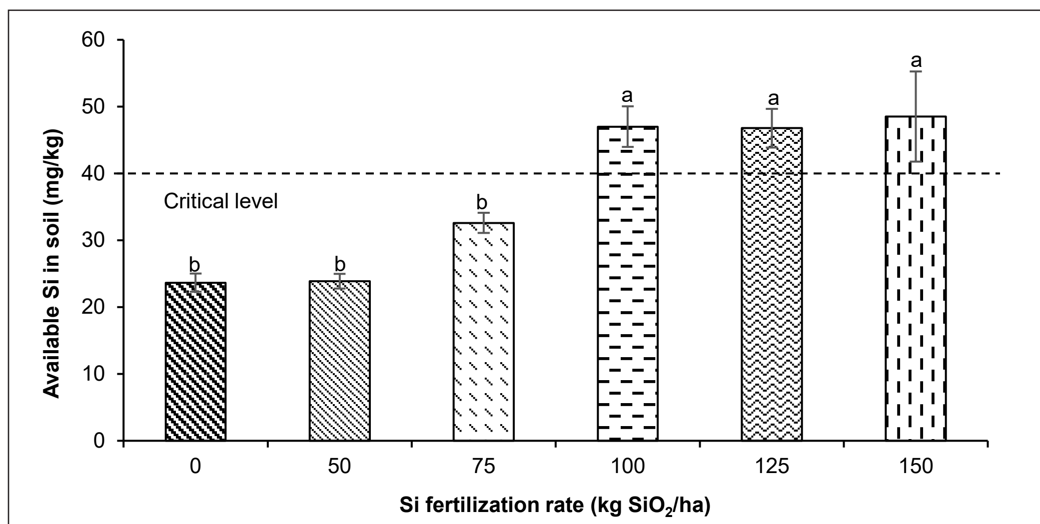
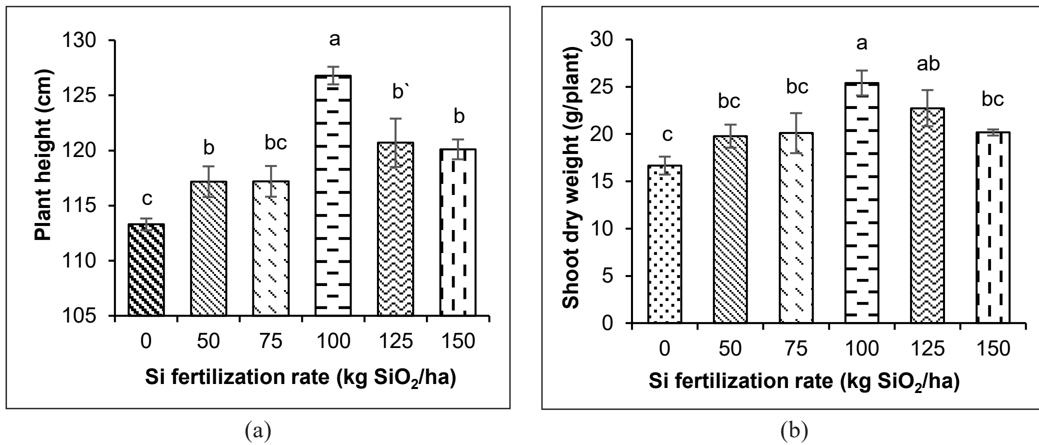



*Figure 1*. Available Si in the soil after harvest for each Si fertilization rate
*Note*. Means with the same letters are not significantly different ($p < 0.05$) using DMRT




(a) (b)

*Figure 2*. (a) Plant height and (b) shoot dry weight at harvesting stage with different rates of Si fertilization
*Note*. Means with the same letters are not significantly different ($p < 0.05$) using DMRT

## Effects of Different Rates of Silicon on Biosynthesis of Chlorophyll

The biosynthesis of chlorophyll pigment was significantly affected by Si fertilization. The gradual and significant increase in total chlorophyll content was observed with the increasing rate of Si applied up to the rate of 125 kg $SiO_2$/ha and then decreased with a further increase of Si (Figure 3). The addition of Si fertilizer has increased the total chlorophyll content by 35–65% over the control, and the maximum was recorded in 125 kg $SiO_2$/ha treated plants. However, it was not significantly different from 100 kg $SiO_2$ /ha treated plants.

## Effect of Different Rates of Silicon on Tissue Silicon Concentration

The significant differences ($p < 0.05$) in Si concentration were observed in tissues

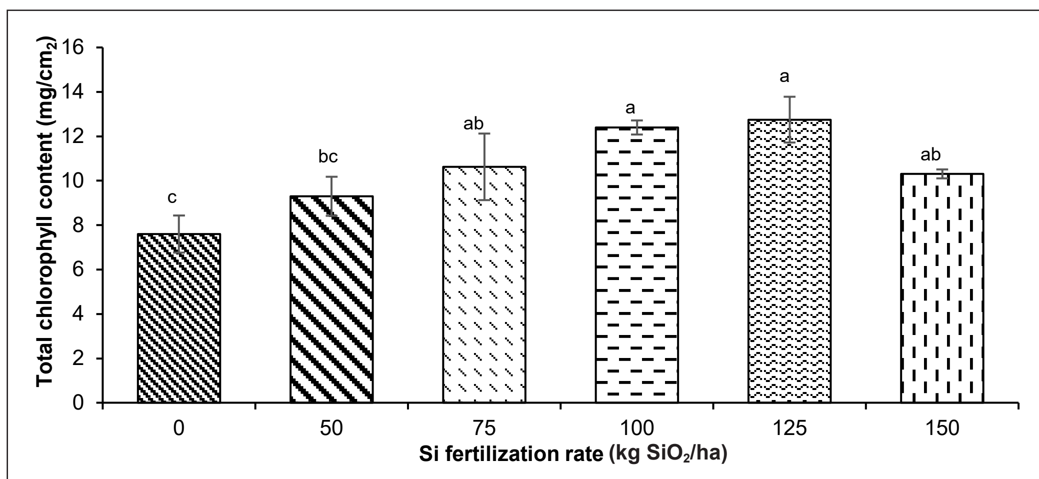



*Figure 3*. Total chlorophyll content at the flowering stage with different rates of Si fertilization
*Note*. Means with the same letters are not significantly different ($p < 0.05$) using DMRT

raised with different Si rates (Figure 4). There were no significant differences between Si rates of 100 and 125 kg $SiO_2$/ha as well as between 50 kg $SiO_2$/ha and control on tissue Si concentrations. The lowest Si concentration of 54.5 µg/100 mg was observed in the control, while the highest Si concentration of 395.3 µg/100 mg was observed at the Si rate of 100 kg $SiO_2$/ha, which was statistically similar to the rate of 125 kg $SiO_2$/ha. Almost 2, 3.4, 7.25, 6.7, and 5.2 folds more Si was accumulated in the shoot tissues of plants grown under 50, 75, 100, 125, and 150 kg $SiO_2$/ha treatments, respectively when compared to the control.

### Effects of Different Silicon Rates on Silicon Uptake

In Bw 367, significant differences were observed under Si uptake in varied levels of Si fertilization (Figure 5). The Si uptake ability of a plant is a genotypic character

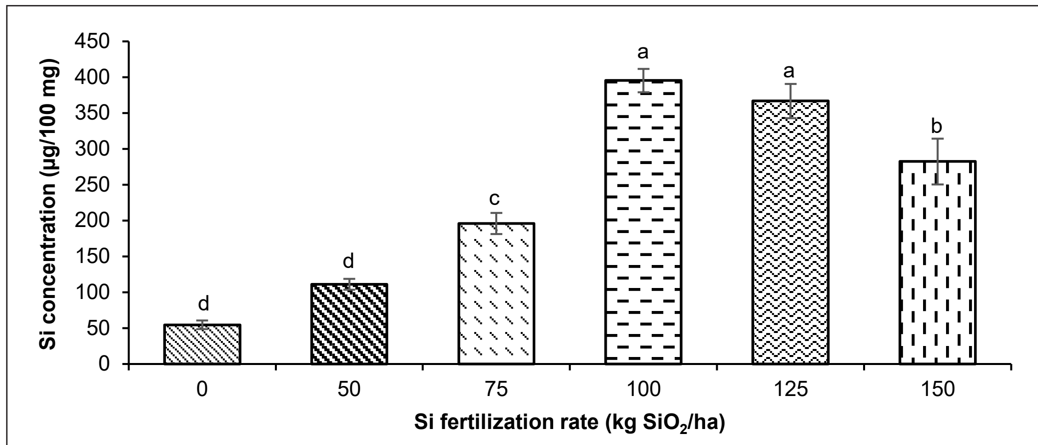



*Figure 4*. Silicon concentration in tissues at the harvesting stage with different rates of Si fertilization
*Note*. Means with the same letters are not significantly different ($p < 0.05$) using DMRT

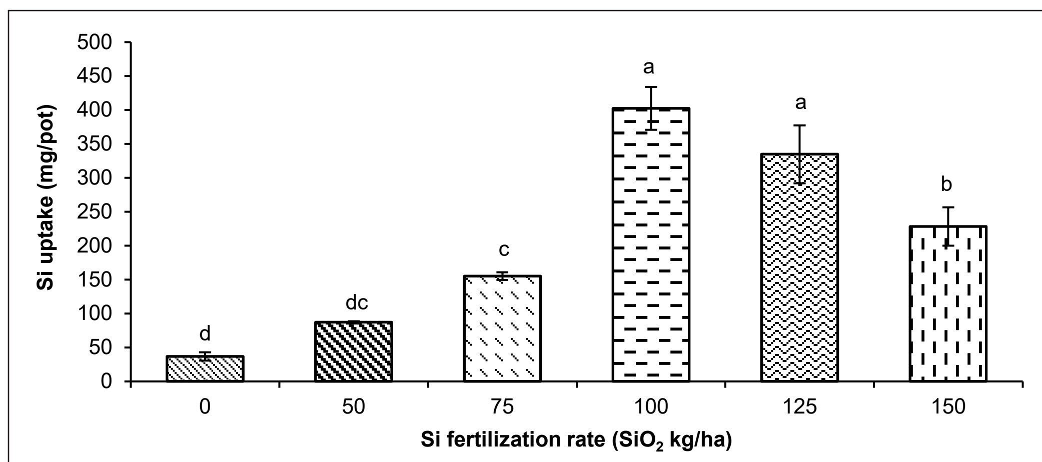



*Figure 5*. Total silicon uptake at the harvesting stage with different rates of Si fertilization
*Note*. Means with the same letters are not significantly different ($p < 0.05$) using DMRT

(Rupasinghe et al., 2021; Swain et al., 2016). A gradual and several folds increase in relative Si concentration was observed in shoots with increasing rates of Si supplied in the root medium. However, Si uptake was significantly decreased when applying the highest rate of Si. The highest amount of Si uptake (402.4 mg/pot) was observed in plants treated with 100 kg $SiO_2$/ha, and it was statistically similar to plants treated with 125 kg $SiO_2$/ha.

**Effects of Different Silicon Rates on Phosphorous Uptake**

The current results found that the P uptake in rice plants significantly varied depending upon the variation of Si application (Figure 6). It was noticed that P accumulation was significantly higher in Si fertilized plants than in Si non fertilized plants. Further, a significant increase in P accumulations was observed with the increased level of Si application. Compared to the control, plants treated with the 100 kg $SiO_2$/ha treatment resulted in a 107% increase in P uptake, indicating that rice crops in Si-fertilized soil had a greater response to P nutrition.

The present study showed that plants grown using 100 and 125 kg $SiO_2$/ha Si treatments recorded a significantly high yield have enhanced accumulation of Si and P in their tissues.

**Effects of Different Silicon Rates on Potassium Uptake**

Different Si rates had a significant ($p < 0.05$) effect on K uptake by rice shoots (Figure 7). An increase in applied Si rate resulted in increased K absorption and accumulation in shoots, consequently increasing the dry weight. However, K was mostly accumulated in the shoots of Si-treated plants when applied at a rate of 100 kg $SiO_2$/ha, which was approximately 1,912 mg/pot and was twofold higher than in Si-untreated plants. However, an increase of Si at 125 and 150 kg $SiO_2$/ha rates tend to decrease the K uptake but is not significantly different with 100 kg $SiO_2$/ha application.

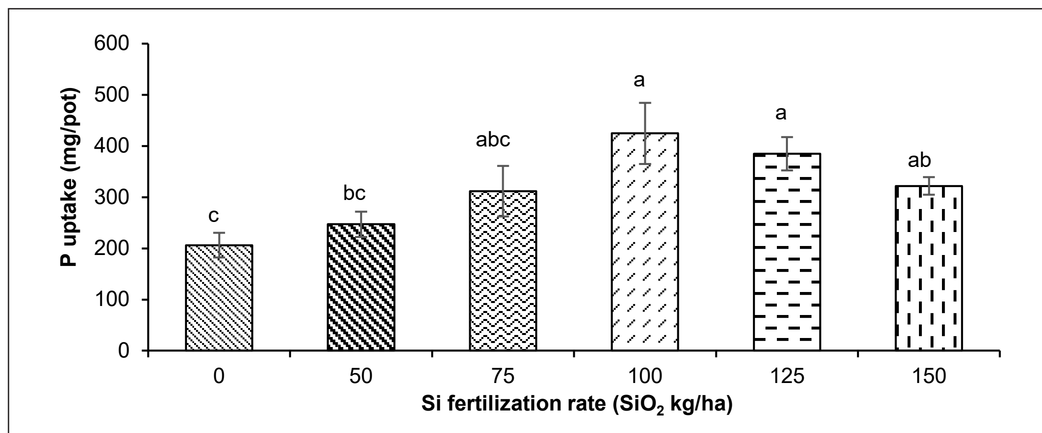



*Figure 6*. Total phosphorous uptake at the harvesting stage with different rates of Si fertilization
*Note*. Means with the same letters are not significantly different ($p < 0.05$) using DMRT

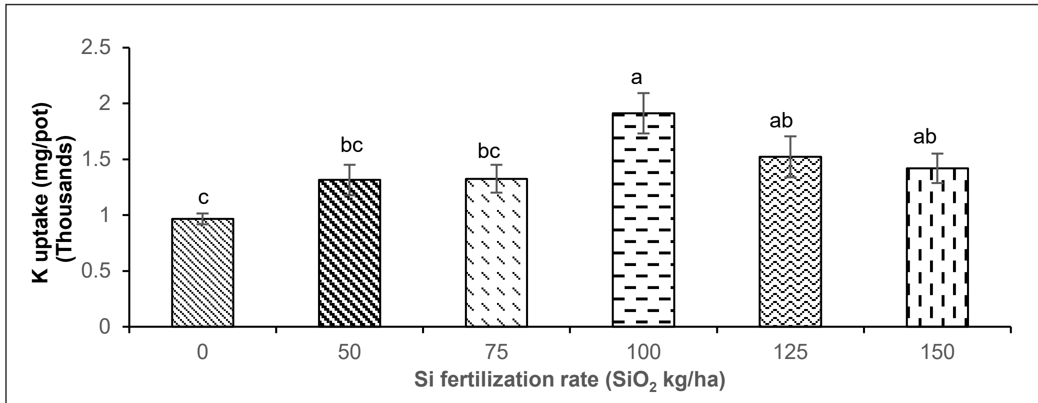



*Figure 7*. Total potassium uptake at the harvesting stage with different rates of Si fertilization
*Note*. Means with the same letters are not significantly different ($p < 0.05$) using DMRT

**Effects of Different Silicon Rates on Magnesium Uptake**

Magnesium uptake of rice plants exposed to the different rates of $SiO_2$ (50-150 kg $SiO_2$/ha) was significantly increased ($p < 0.05$) in comparison to the control. Applied Si rate increased Mg uptake gradually but then decreased at the highest rate of Si concentration applied (Figure 8). Mg uptake increased from 11% to 75% in Si fertilized plants compared to the control. The highest amount, 291.72 mg/pot of Mg, was accumulated in plants, which received Si at the rate of 100 kg $SiO_2$/ha, and it was statistically similar to the rate of 125 and 150 kg $SiO_2$/ha.

**Effect of Silicon on Yield Attributes of Rice**

A productive tiller is one of the important yield-determining parameters in rice. However, in this research, regardless of

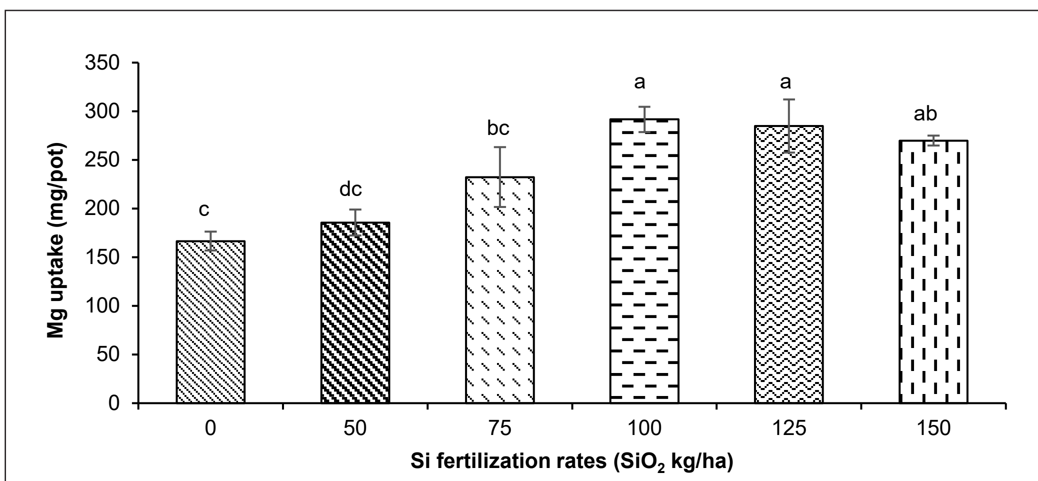



*Figure 8*. Total magnesium uptake at the harvesting stage with different rates of Si fertilization
*Note*. Means with the same letters are not significantly different ($p < 0.05$) using DMRT

the six different Si fertilizer treatments, rice plants produced the statistically same number of productive tillers (Table 2).

The number of filled grains/panicle is another yield-determining component in rice, and it was significantly affected by the application of Si (Table 2). All the Si-treated rice plants yielded a significantly higher amount of filled grains than the control plants. This study showed that filled grains/panicle increased with increasing of Si rate up to 125 kg $SiO_2$/ha. The highest mean number of filled grains/panicle (343) was produced by applying Si at the rate of 100 kg $SiO_2$/ha, closely followed by the rate of 125 kg $SiO_2$/ha (340). The filled grains/panicle increased with the application of Si fertilizer at 100 kg $SiO_2$/ha over the control (0 kg $SiO_2$/ha) was about 51%, and it was a remarkable improvement in Si application which impressively indicated the yield improvement.

Silicon has a positive effect on the grain filling of rice, resulting in a reduced number of unfilled grains/panicle. According to the results, 30% of unfilled grain reduction was observed by applying Si fertilizer at the rate of 100 kg $SiO_2$/ha, which was statistically similar to 125 kg $SiO_2$/ha. The highest number of unfilled grains/panicle of 70 was developed in the Si untreated plants confirming the positive effect of Si on the formation of filled grains. Among the applied levels of Si, 50, 100, and 125 kg $SiO_2$/ha recorded the lowest number of unfilled grains, while filled grains were highest only in the rates of 100 and 125 kg $SiO_2$/ha.

Significant differences in the total number of grains/panicle were seen in response to the enforced Si treatments. Total grains/panicle of 100 and 125 kg $SiO_2$/ha fertilized plants recorded the significantly highest number of grains, amounting to 392, following the same pattern as the number of filled grains/panicle. Another important yield component in rice is the 1,000-grain weight. However, it is more specific to the genotype than a response to fertilization (Cox & Smith, 2019). As demonstrated in Table 2, this study also found that the applied Si on 1,000-grain weight was non-significant over the control.

Table 2
*Yield components of rice with different rates of silicon fertilization*

| Si fertilization rate (kg $SiO_2$/ha) | Productive tillers/panicle | Filled grains/panicle | Unfilled grains/panicle | Total grains /panicle | 1,000-grain weight (g) |
|---|---|---|---|---|---|
| 0 | 9 ± 0a* | 226 ±11c | 70 ± 5a | 296 ± 12d | 14.7 ± 0.27a |
| 50 | 8 ± 0.57a | 259 ± 11bc | 49 ± 3c | 308 ± 8dc | 14.23 ± 0.23a |
| 75 | 8 ± 1.2a | 292 ± 6b | 63 ± 4ab | 356 ± 4ab | 14.15 ± 0.52a |
| 100 | 8 ± 0.33a | 343 ± 10a | 49 ± 8c | 392 ± 11a | 14.57 ± 0.03a |
| 125 | 8 ± 0.88a | 340 ± 10a | 49 ± 5c | 389 ± 14a | 14.23 ± 0.43a |
| 150 | 9 ± 0.33a | 293 ± 15b | 52 ± 6bc | 345 ± 16bc | 14.87 ± 0.28a |

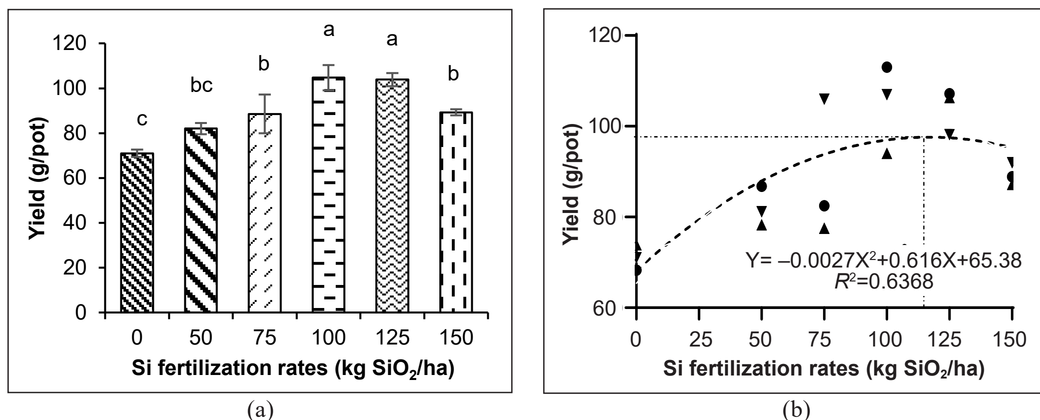
*Note*. Means with the same letters across the column are not significantly different ($p < 0.05$) using DMRT
* ± value indicates the standard error of the mean (n=3)

**Effect of Silicon on Grain Yield of Rice**

The grain yield of the Bw 367 genotype was significantly affected by the varied level of the added Si (Figure 9a). The highest pot yield (104.6 g/pot) was recorded in the plants fertilized with 100 kg $SiO_2$/ha, and it was on par with the pot yield of plants fertilized with 125 kg $SiO_2$/ha rate. The relative grain yield of 100 kg $SiO_2$/ha treatment was 47% higher than the control. Same as the other tested parameters, the highest rate of Si yielded a significantly lower yield than that of the 100 and 125 kg $SiO_2$/ha treatments.

**Correlation Analysis**

The correlation study was used to find out the relationship between the tested parameters (Table 3). Plant available Si in the soil was increased with the applied Si rates (Figure 1), and rice plants adsorbed more Si when Si fertilizer was supplied




(a) (b)

*Figure 9*. Grain yield with different rates of Si fertilization: (a) Yield at different Si rates; and (b) Si rate required for the optimum level of the yield of Bw 367 genotype
*Note*. Means with the same letters are not significantly different ($p < 0.05$) using DMRT

Table 3
*Correlation between available silicon in soil and other parameters*

| Parameter | SiU | PU | KU | MgU | Chl | SDW | AvSi |
|---|---|---|---|---|---|---|---|
| PU | 0.46 | | | | | | |
| KU | 0.59* | 0.92** | | | | | |
| MgU | 0.49* | 0.89** | 0.82** | | | | |
| Chl | 0.77** | 0.88** | 0.82** | 0.80** | | | |
| SDW | 0.63* | 0.81** | 0.90** | 0.68** | 0.83** | | |
| AvSi | 0.57** | 0.73** | 0.64** | 0.74* | 0.78** | 0.62* | |
| Y | 0.71** | 0.82** | 0.86** | 0.83** | 0.88** | 0.84** | 0.76** |

*Note*. * = Significant at $p$ <0.05; ** = Significant at $p$ < 0.01; SiU = Silicon uptake; PU = Phosphorous uptake; KU = Potassium uptake; MgU = Magnesium uptake; Chl = Chlorophyll content; SDW = Shoot dry weight; AvSi = Available silicon; Y = Yield

(Figure 4). Plant available Si in soil was strongly correlated with plant P uptake (0.73**), Mg uptake (0.74**), chlorophyll content (0.78**), and yield (0.76**) and moderately correlated with K uptake (0.64**) and Si uptake (0.57**). It is obvious that Si fertilization increases the available plant Si in the soil, and it improves the soil nutrient uptake as well as biosynthesis of chlorophyll in tissues. All these parameters are strongly correlated with the yield. Moreover, Si uptake was strongly correlated with shoot dry weight ($r$ = 0.63**), yield (0.7**) and chlorophyll content (0.77**). However, the correlation between Si uptake and P uptake was not significant.

## DISCUSSION

Various rates of $SiO_2$ fertilization resulted in a considerable rise in Si concentration in tissues as well as Si uptake. Increased Si uptake with Si fertilizer application could be attributed to the increased level of available Si in the soil and improved root systems, which could encourage the plant to absorb more Si from the soil solution (Pati et al., 2016). Despite the abundance of Si in the soil, plant-available Si in the soil solution is limited (Jawahar & Vaiyapuri, 2013). Therefore, exogenous Si application is required. Silicon fertilizer is applied to enhance the availability of monosilicic acid for plant uptake. However, available Si in soil was not significantly increased with Si fertilization beyond 100 kg $SiO_2$/ha. Silicon concentration in soil at 2mM Si is potentially the concentration of Si at which polymerization begins even in soil (Fortunato et al., 2015). As the rate of Si addition increases, the monosilicic acid concentration begins to polymerize, forming polysilicic acid, which plants cannot absorb (Fortunato et al., 2015). Similarly, in this study, Si applied rates higher than 100 kg $SiO_2$/ha may have increased the Si concentration in soil by more than 2mM, leading to polymerization of Si. It could be the reason for no significance of AvSi concentrations even at higher Si application rates. Hence, the application of Si higher than the rate of 100 kg $SiO_2$/ha seems to be unproductive.

Endogenous Si in rice plants helps to increase the plant growth parameters, including plant height and shoot dry weight, as found in past research evidence (Cuong et al., 2017; Kim et al., 2012; Rupasinghe et al., 2021). The increase in plant height could be attributed to the rapid elongation of the stem caused by Si (Peera et al., 2014). Further, the synthesis of total chlorophyll (chlorophyll *a* and *b*) content was enhanced with the applied Si and thereby increased the photosynthetic capacity of the rice plant. It may be due to the keeping of the leaf blade erect by the accumulated Si, thereby preventing the mutual shading of leaves and improving the light interception (Mauad et al., 2003). In addition, the application of Si significantly improved the uptake of P, K, and Mg alone with Si in the shoots of the rice plant, which is consistent with previous observations as well (Crooks & Prentice, 2017; Pati et al., 2016). Finally, it may be due to the enhanced nutrient availability in the soil. Soil Si enhances the nutrient availability in the soil and stimulates root

growth (Swain & Rout, 2018), enabling increased nutrient absorption. With the presence of Si, potassium ions ($K^+$) are adsorbed onto the silica surface, increasing the availability for plant absorption. The increase in P uptake is due to the increased fertilizer use efficiency, which makes more P available for plant uptake (Subramanian & Gopalswamy, 1991). Accordingly, Si fertilizer improves plant growth caused of enhanced photosynthetic capacity and nutrient uptake. Magnesium is a constituent of chlorophyll molecule as a central atom in the structure. This study observed that the addition of Si improved chlorophyll synthesis, which could be resulted from the increased Mg uptake. In contrast, an inadequate level of Mg in photosynthesis reduces the dry matter assimilation in plant tissues (Tränkner et al., 2018).

Rice grain yield is influenced by the tillering capability of the plant, which is associated with the number of panicles per unit area (Efisue et al., 2014). However, Si fertilization did not affect the number of productive tillers per plant, which contradicts Hoseinian et al. (2020) and Pati et al. (2016), who found a considerable increase in productive tillers with Si application. The possible reason for the non-significance effect on a number of tillers is a genetic character, which hardly changed with the environmental effects.

In rice, key yield components are the total number of grains/panicle, the number of filled grains/panicle, the number of unfilled grains/panicle, and the 1,000 grains weight. Therefore, increasing these components and decreasing the number of unfilled grains/panicles have a direct influence on rice production. We discovered a positive response in the number of grains per panicle to applied Si, which agrees with Lavinsky et al. (2016), who mentioned that Si is an important factor in increasing the number of grains per panicle in rice. The grain yield of Si-treated plants was increased due to growth, photosynthesis capacity, and balanced nutrition improvements. Further, Matoh et al. (1991) observed that accumulating a large amount of Si in plant tissue reduces the loss of water, which helps to ease water stress in the plant.

A significant reduction in unfilled grain was observed as well in this study, and it could be attributable to better nutrition, increased metabolic activity, reduction in moisture stress, or a combination of these factors.

A positive response of filled grains per panicle to Si fertilizer was noticed in this study. This finding is consistent with that of Jawahar et al. (2015), who observed that Si fertilizer promotes carbohydrate assimilations in panicles, resulting in a higher number of filled grains per panicle. The number of filled grains in 100 kg $SiO_2$/ha treated plants was the highest owing to the greatest amount of Si uptake and dry matter assimilation at this Si applied rate.

The weight of 1,000 grains is a genotypic trait (Huang et al., 2013); hence it is less likely to be influenced by other factors. The Si fertilizer also had a non-significant influence on grain weight. These results are comparable to those of Mobasser et al. (2008), who found that adding Si to rice does not affect the 1,000 seed weight;

however, it is contradictory to the findings of Cuong et al. (2017) and Mahendran et al. (2021), who elaborated that using Si fertilizer enhanced rice grain weight. Even at the grain filling stage, Si treatment did not result in a substantial increase in grain size or weight (Kim et al., 2012), which further confirmed our results. With the provision of Si to the rice plant, Si has translocated inside the hull of the seeds as well as the kernel but may not be deposited continuously. Therefore, it would be advantageous to keep the grain size at its specific small size, especially in small grain genotypes like Bw 367, to maintain the consumers' preference. The results were consistent with Pati et al. (2016) and Prakash et al. (2011). The increases in grain yield in this study could be due to the positive effect of Si in increasing growth and yield determining characters, such as number of grains/panicle, number of filled grains/panicle, and reduction in unfilled grains/panicle. Simulation using a quadratic function suggests that the Si fertilization rate for optimum grain yield of rice was 115 kg $SiO_2$/ha (Figure 9b).

Most of the tested parameters recorded their significantly highest values at the rate of 100 kg $SiO_2$/ha. Applying Si above this rate was not beneficial for further improvement in growth and yield attributes. However, any toxic effect of Si was also not observed in the excess application of Si fertilization. It could be due to the polymerization of silicic acid. In the xylem, Si presents as silicic acid. However, when the Si concentration is higher than 2mM, silicic acid is polymerized to silica gel. Further, the polymerization process is aggravated by water loss (Ma & Yamaji, 2006).

## CONCLUSION

Applied Si improved the tested *indica* rice plant growth, nutrient uptake, and yield. Therefore, all paddy lands need to be fertilized with Si prior to establishing the next crop to maintain the required level of available Si in soil for healthy and high crop production.

It can be recommended that applying Si at the rate of 115 kg $SiO_2$/ha was the optimum rate of Si fertilizer, which can provide the maximum yield under the glasshouse condition. Furthermore, the different Si fertilizer rates were tested on high-yielding rice genotypes. Thus, the optimal Si rate identified can be included in the general fertilizer recommendation for paddy cultivation. However, this value should be further tested in real field conditions.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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*Review Article*

# Host Range and Control Strategies of *Phytophthora palmivora* in Southeast Asia Perennial Crops

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## ABSTRACT

*Phytophthora palmivora* is a destructive plant pathogenic oomycete that has caused lethal diseases in a wide range of hosts. It is a pan-tropical distributed pathogen that can infect plants at all growth stages. Extensive studies have linked *P. palmivora* to severe diseases in several crops, such as black pepper, rubber, cocoa, and durian, causing global economic losses. This review covers the following topics in depth: (i) *P. palmivora* as phytopathogen; (ii) identification and infection mechanism in rubber, cocoa, and durian; and (iii) management and control applied for *P. palmivora* diseases. Effective management strategies were studied and practiced to prevent the spread of *P. palmivora* disease. Genetic resistance and biocontrol are the best methods to control the disease. A better understanding of *P. palmivora* infection mechanisms in our main crops and early disease detection can reduce the risk of catastrophic pandemics.

*Keywords*: Cocoa, disease control, durian, *Phytophthora palmivora*, rubber

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## INTRODUCTION

Food security has become a global issue affecting the agricultural revenue of many countries. The rising costs of overcoming challenges have driven up the price of staple foods. Pests and diseases are important biotic factors that cause over 20% to 40% of agricultural productivity, affecting the

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global economy (Oerke, 2006). Agriculture provides endless wealth and nutrition to tropical people. Crops (such as cocoa, coconut, and rubber), fruits (such as durian, jackfruit, papaya, and pineapple), and root crops (such as potato and taro) were initially planted for domestic consumption. These crops have recently gained popularity in tropical regions and are now one of the country's primary sources of agricultural income. Lethal diseases caused by the *Phytophthora* genus have always posed a significant threat to crop yield and have a global impact on the agricultural industry.

*Phytophthora* is a genus of destructive plant-pathogenic oomycetes responsible for economic losses in agriculture and ecosystem damage worldwide. *Phytophthora* means "plant destroyer" and was initially classified within the fungi kingdom until a phylogenetic study revealed the differences between oomycete and true fungi (Vanegtern et al., 2015). More than 150 *Phytophthora* species have been identified and could cause devastating diseases in annual and perennial crops in tropical and temperate regions (Yang et al., 2017). *Phytophthora* species vary greatly in host specificity as they could infect multiple plants from different families, while others are host-specific (Latifah et al., 2018). *Phytophthora palmivora* is highly virulent and can have a significant impact on the production of valuable crops, as well as the economies of the leading agricultural producing nations (Table 1).

Table 1
*The total production of rubber, cocoa, durian, and oil palm in selected Southeast Asian countries and estimated average loss caused by* Phytophthora palmivora *diseases*

| Crop | Country | Production (Metric tons) | Disease loss (%) | References |
|---|---|---|---|---|
| Rubber | Malaysia | 705,292[a] | 30-50 | Sunpapao and Pornsuriya (2014) |
| | Indonesia | 3,801,631[a] | 30-50 | |
| | Thailand | 5,335,134[a] | 30-50 | |
| | Philippines | 475,840[a] | 30-50 | |
| | Vietnam | 1,306,412[a] | 30-50 | |
| Cocoa | Malaysia | 1108[a] | 20-30 | Hebbar (2007) |
| | Indonesia | 734,796[a] | 20-30 | |
| | Thailand | 125[a] | 20-30 | |
| | Philippines | 9358[a] | 20-30 | |
| | Vietnam | NA | NA | |
| Durian | Malaysia | 390,635.44[c] | 20-30 | Drenth and Sendall (2004) |
| | Indonesia | 128,376[b] | 20-30 | |
| | Thailand | 1,017,097[e] | 20-30 | Drenth and Sendall (2004) |
| | Philippines | 79,280[d] | 20-30 | Drenth and Sendall (2004) |
| | Vietnam | 434,179[f] | 10 | Thao et al. (2020) |

*Note*. [a]Food and Agriculture Organization of the United Nations (FAO) (2019); [b]Ministry of Agriculture of The Republic of Indonesia (MOA) (2016); [c]Department of Agriculture (DOA) (2019); [d]Philippine Statistics Authority (PSA) (2019); [e]Thongkaew et al. (2021); [f]Thao et al. (2020)

## MODE OF ACTION OF *PHYTOPHTHORA PALMIVORA*

*Phytophthora* species use a hemibiotrophic lifestyle, infecting and feeding on living cells during the biotrophic phase, then on dead or dying cells during the necrotrophic phase to colonize and feed on living host cells. Extensive research suggests this lifestyle is a driving force underpinning the success of *Phytophthora* species. *Phytophthora* reproduces sexually via dispersal spores (sporangia and zoospores) and asexually via resting spores (oospores and chlamydospores) (Figure 1) (Erwin & Ribeiro, 1996; Judelson & Blanco, 2005; Perrine-walker, 2020). Sporangia and zoospores help *Phytophthora* species to spread and escape from the deteriorating environment, whereas chlamydospores are reserved and remain viable in the soil for a long time, waiting for favorable and conducive conditions for sporulation and dispersal (Butubu, 2016).

In favorable humid conditions, the flagellated zoospores swim chemotactically or electrostatically onto plant tissue and encyst in soil water or thin water film chemotactically or electrostatically (Butubu, 2016; Widmer, 2014). The germ tube then emerged from the mononucleate cyst. During cyst germination, the nucleus in the cyst undergoes closed mitosis and travels actively in the bilateral movement. The ability of the nucleus to form a hydrodynamic shape helps to maintain long distances and free flow movements within the elongated hyphae. (Evangelisti et al., 2019).

Unlike fungi, oomycetes lack melanins and septins needed for appressorium formation. Instead, *Phytophthora* penetrates its hosts by utilizing hyphal slicing, also known as the naifu mechanism, which cuts through the host surface at an oblique angle with minimal energy. This strategy facilitates the oomycete invasion through actin-mediated polar force application that

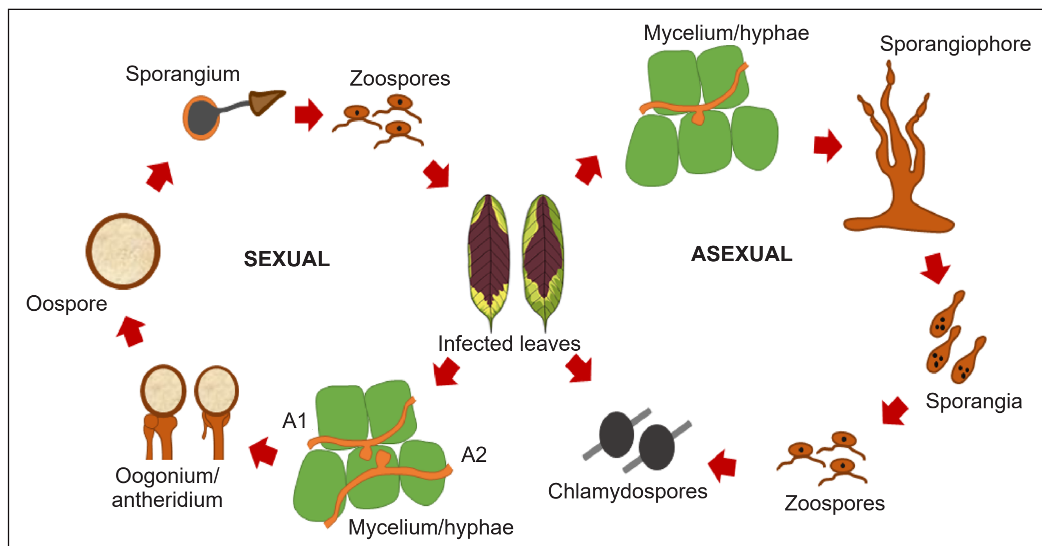


*Figure 1*. The life cycle of *Phytophthora palmivora*

helps disrupt the targeted host's cytoskeletal structure. The turgor pressure produced and the disruption of the cytoskeleton structure will allow the hyphae to invade through the crack along the direction of the oblique angle, initiating the host surface fracture (Bronkhorst et al., 2021). These discoveries of the *Phytophthora* infection mechanism can be a good initiative to develop durable resistance in the plant by interrupting the crucial steps during nuclear movement and hyphal slicing.

## FACTORS CONTRIBUTING TO *PHYTOPHTHORA PALMIVORA* INFECTION

In the intervening years, extensive studies have explored the driving factors of *P. palmivora* in invading and colonizing their hosts. The hot-humid tropical climate in Malaysia and other Southeast Asian countries with high annual rainfall is ideal for developing and spreading *P. palmivora* diseases (Lee & Lum, 2004). A high humidity area or even a splash of water will make it easier for the flagellated zoospores to swim and infect the hosts. On top of that, the geographical area of the infected crops is also one contributing factor. For instance, flat open areas and lower areas of the fields where irrigation water may accumulate are favorable for *Phytophthora* species infection (Pscheidt & Ocamb, 2022).

The transmission of *P. palmivora* is most likely accelerated by numerous biotic and abiotic factors, such as extreme weather events that play a crucial multifaceted role in various ecological communities and interactions (Mohamed Azni et al., 2019). Global warming poses significant impacts on plant and animal diseases, thus threatening food security (Mariette et al., 2016). The exceptional high temperature will cause heat stress on plants and significantly accelerate soil water evaporation, thus decreasing soil moisture content making the soil drier, and eventually causing soil erosion. Soil erosion causes excessive nutrient leaching, decreasing resistance to plant pathogen infection, and causing new strains and diseases to emerge (Paterson et al., 2013).

Drastic climate changes have high impacts on the modification and evolution of an individual, population, species, or even ecosystems (Bellard et al., 2012). In host-pathogen interaction, climate change exerts significant influence on the pathogen evolutionary adaptation pattern and host resistance by affecting important stages of the life cycles of either or both hosts and pathogens, as well as their reproductive mechanisms, dispersal ability, and their interactions with other biotic and abiotic factors in the environment (Addison et al., 2013; Dysthe et al., 2015; Eastburn et al., 2011; Mboup et al., 2012; Urban Mark et al., 2012). Some hosts might struggle to adapt well to the changing environment, thus allowing the pathogens that could evolve rapidly and adapt to the abrupt changes in thermal environments to invade easily (Paterson et al., 2013). *Phytophthora* species that are well known for their high adaptability skills are not exceptional. Temperature acclimation notably enhances *Phytophthora*'s fitness and genetic adaptation at both low and high

temperatures by increasing their colony size and aggressiveness (Wu et al., 2020). Additionally, Paterson (2020) predicted that *P. palmivora* can still infect all the oil palms that survived extreme global warming by 2050 and, even worse, cause the emergence of *P. palmivora* diseases in oil palm plantations in Malaysia and Indonesia if there are no extra protective measures taken.

On top of that, global migration is also known as one of the crucial key points that cause plant pathogen evolution. Wang et al. (2020) suggested that *P. palmivora* is a native pathogen of South American hosts that has spread and diversified in Southeast Asia. Single colonization of *P. palmivora* on cocoa was responsible for the global pandemic of black pod rot disease in cocoa and the migration of *P. palmivora* infection to other hosts (Wang et al., 2020). Global trade and migration have exposed plant pathogens to new hosts, facilitating host jumps (Zhan et al., 2015).

## HOST RANGE OF *PHYTOPHTHORA PALMIVORA*

*Phytophthora palmivora* is a threatening tropical and subtropical oomycete that infects a wide range of hosts worldwide. Recent reports suggest that *P. palmivora* had attacked over 170 species of agricultural and horticultural plants, causing huge production losses (Drenth & Guest, 2013). In addition to attacking different hosts, *P. palmivora* can infect multiple plant tissues such as roots, stems, flowers, leaves, and fruits of individual plant species - all at once.

### Rubber (*Hevea brasiliensis*)

The rubber tree (*Hevea brasiliensis*) is a tropical crop native to South America cultivated for its latex, which is known as the primary source of rubber in the rubber industry. Until the late 1980s, Malaysia was the world's largest rubber producer before Indonesia and Thailand took over (Balsiger et al., 2000; Ratnasingam et al., 2011). Rubber plantations require well-distributed high annual rainfall, deep and well-drained soils, stable high temperatures, and continuous moisture throughout the year to produce high-quality latex. A young rubber tree can tolerate temperatures as low as 15 °C for extended periods. However, prolonged exposure to low temperatures and dry conditions for more than 2 to 3 months will reduce the quantity and quality of latex produced.

The environment where the rubber is planted makes them vulnerable to diseases caused by *P. palmivora*. In Thailand, leaf fall epidemics happen during high rainfall (June to December), when rain splashes spread pathogen zoospores from infected leaves onto a trapping panel (Johnston, 1989). *Phytophthora palmivora* causes black stripe and abnormal leaf fall, reducing latex production globally (Verheye, 2010). Both diseases were reported in almost all countries where *H. brasiliensis* were planted, such as Malaysia, Sri Lanka, Myanmar, Indonesia, Thailand, Vietnam, and the Philippines (Drenth & Guest, 2004).

**Infection Mechanism in Rubber.** The infection began with the germination of

resting chlamydospores in infected dried pods or leaves left in the soil or on trees (Drenth & Guest, 2004). The green pods will develop; symptoms include water-soaked dull-grey-colored lesions, a cheesy coating on the pods, and latex oozing. Mycelia penetration and sporangia production developed a cheese coating on the pods. The disease then spreads to the leaves, which are more common on the petioles. The dark brown lesion on the petioles oozes a drop of coagulated latex. While defoliating, infected leaves remain. A water-soaked lesion on the leaf lamina causes the leaves to turn black (Jacob et al., 2006, as cited in Krishnan et al., 2019, p. 35). In favorable climatic conditions, the leaf falls become more severe, especially in susceptible varieties, causing heavy defoliation that will lead to the formation of a leaf carpet covering the entire ground and a significant latex reduction.

To prevent pathogen infection of healthy and unaffected tissues, *H. brasiliensis* synthesizes several anti-fungal compounds. In Thailand, resistant clones (RRIC 100 and BPM 24) had a higher concentration of phenolic compounds than susceptible clones (RRIC 121, RRIM 600, and PB 86), suggesting that the lignin produced from phenolic aldehydes surrounding diseased plant portions improves host resistance (Jayasuriya et al., 2003).

**Cocoa (*Theobroma cacao*)**

*Phytophthora* diseases threaten global cocoa production (Peter & Chandramohanan, 2011). *Phytophthora palmivora* is the primary pathogen responsible for black pod rot, stem canker, seedling blight, chupon wilt, and flower cushion infection in cacao trees (Akrofi et al., 2003; Mcmahon & Purwantara, 2004). However, black pod rot is the most significant factor limiting production spread in the cocoa-growing region, accounting for 10-30% of annual yield losses of cocoa beans worldwide and up to 40% losses in wet and humid conditions (Hebbar, 2007; Purwantara et al., 2004; Vanegtern et al., 2015). An outbreak in nearly every cocoa-producing country, primarily in South America and Southeast Asia, has resulted in annual losses of up to 450,000 metric tons (Wahyudi & Misnawi, 2008). It is also reported to have cost Malaysia over 30% of its annual cocoa production (Alsultan et al., 2019). *Phytophthora megakarya* and *P. palmivora* are black pod rot's most important causal agents worldwide. *Phytophthora palmivora* causes the disease in Asia, Central America, and South America, while both species may exist and can cause black pod rot in West Africa (Widmer, 2014). *Phytophthora palmivora* can infect all age ranges of cacao trees, both young and matured trees, causing multiple diseases, such as seedling blight, leaf blight, stem canker, and black pod rot (Purwantara et al., 2015). Infection of cocoa pods occurs in the two months before ripening, causing the most significant loss. After that, the pathogen can easily spread from the pod husk to the bean's seed coat, causing a total loss (Wahyudi & Misnawi, 2008).

**Infection Mechanism in Cocoa.** The infection of black pod rot began with an early penetration into the waxy cuticle. Then, it progressed to the epidermis, causing a small translucent spot to develop into a brownish lesion. The lesion will darken and expand rapidly, gaining 12 mm in 24 hours. The infection quickly spread throughout the entire pod, causing a severe black lesion in the infected area. In favorable conditions, clusters of white sporangia will gradually spread on the pod surface (Purwantara et al., 2015; Wahyudi & Misnawi, 2008). The authors have further explained that most cacao ripe pods can be recovered from a light infection, but advanced infection causes total loss (Purwantara et al., 2015).

### Durian (*Durio zibethinus*)

Durian (*Durio zibethinus*) is one of Southeast Asia's most valuable tropical fruits. Initially from Peninsular Malaysia and Borneo, it has spread globally to Sri Lanka, Northern Australia, and Hawaii (Honsho et al., 2004). Thailand is the largest producer and export of durian, followed by Malaysia and Indonesia, while the Philippines and Vietnam focus on domestic consumption (O'Gara, Sangchote, et al., 2004; Somsri, 2014). For growth and cultivation, durian prefers a tropical climate with high temperatures and consistent rainfall of over 2,000 mm per year (Somsri, 2008).

A major threat to durian production, durian canker caused by *P. palmivora,* has caused devastating economic losses estimated at US$2.3 billion in five Southeast Asian countries: Malaysia, Indonesia, Thailand, the Philippines, and Vietnam (Drenth & Sendall, 2004). The death of durian trees due to durian stem canker was first reported in Penang, where *P. palmivora* was successfully isolated from the main trunk, and canker-like symptoms were observed on the affected trees (Thompson, 1934). In addition, *P. palmivora* causes patch canker, root rot, fruit rot, and leaf blight in durian at all stages of growth (Figure 2) (Lim & Luders, 1998).

**Infection Mechanism in Durian.** *Phytophthora palmivora* thrives in the tropical hot-humid conditions where durian is grown. Furthermore, heavy clay soil and poor drainage will promote the proliferation of the devastating disease in durian, as durian roots are highly sensitive to standing water and susceptible to root rot caused by *P. palmivora* (Somsri, 2008). Besides, the durian leaf surface features help *P. palmivora* pre-penetrate into the host (O'Gara, Sangchote, et al., 2004). Trichomes are found on the abaxial leaf side, petiole, young stem, and fruit of *D. zibethinus*. Three distinct types of trichomes are found on durian leaves (i) glandular trichomes that are not lignified; (ii) stellate trichomes that are not lignified to varying degrees; and (iii) peltate trichomes that are heavily lignified and form the external layer, giving the abaxial surface a silver to golden hue (Husin et al., 2018).

The motile zoospores of *P. palmivora* bind randomly and individually onto the adaxial side of durian leaves, which have a continuous cuticle with no stomata or

trichomes. The abundant and overlapping peltate trichomes on the abaxial side of the leaf trapped a greater proportion of *P. palmivora* spores. Under favorable environmental conditions, *P. palmivora* can bind, produce extensive hyphae, and re-sporulate, thus completing the life cycle on infected durian tissue within eight hours post inoculation (Vawdrey et al., 2005).

In early penetration, *P. palmivora* can directly penetrate the cuticle and epidermis of the adaxial side of durian leaves, as well as the trichome-free area between the fruit spines. To date, there is no recorded successful penetration of *P. palmivora* through the heavily lignified peltate trichomes. However, an attempted penetration was marked by appressoria-like swellings and dissolution of the trichome surface at the attachment area. Unsuccessful penetration attempts usually result in hyphal branch formation on another infection site. A single zoospore will repeat this process until the infection is successfully established. If not, the well-developed hyphae will grow over the edge of the trichome across the leaf surface and spread infection into the host tissue via open stomata (O'Gara, Sangchote, et al., 2004). Once the penetration and infection are established, *P. palmivora* will rapidly colonize the entire leaf lamina, and lesions will appear within two days post-inoculation, ranging from dark brown with a distinct margin to the water-soaked light grey lesion. *Phytophthora palmivora* will proliferate within the host, releasing new sporangia into the environment via stomata or epidermis eruption. As a result, severely infected durian fruits have a whitish bloom (O'Gara, Sangchote, et al., 2004). In fruit, the stylar is the most infected part because rainwater dries slowly and concentrates the spores. When spores land on the fruit surface in favorable conditions, they can infect 50% of the fruit in seven hours and 100% in 17 hours (Siriphanich, 2011).

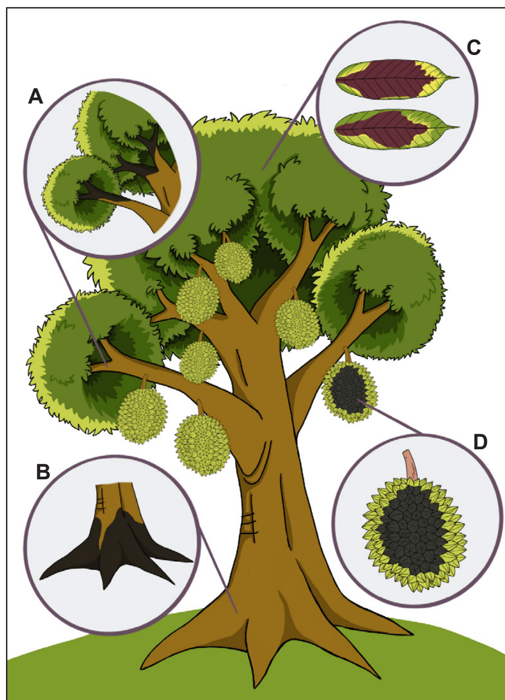


*Figure 2*. Symptoms caused by *Phytophthora palmivora* in durian: A) patch canker; B) root rot; C) leaf blight; and D) fruit rot

## Oil Palm (*Elaeis guineensis*)

Oil palm (*Elaeis guineensis*) is an economically important crop originating in Africa, with Malaysia and Indonesia being the largest producers worldwide (85%). Malaysia is currently earning RM 46.12 billion (US$11.33 billion) in palm oil export revenue to India and European Union markets (Kushairi et al., 2018). Malaysian

palm oil production began commercially as early as 1917 (Ommelna et al., 2012). The crop is also largely planted in Latin American countries such as Colombia, Ecuador, Suriname, Brazil, Costa Rica, and Panama (Rocha et al., 2005). Following Indonesia, Malaysia, Thailand, and Nigeria, Colombia is the fifth largest oil palm producer with an estimated planting area of 450,000 ha and produces more than 30% of America's palm oil (Sundram & Mohamed Azni, 2017; Torres et al., 2016). However, several global diseases hamper the rapid expansion of oil palm cultivation. The oil palm industry in South Africa and Central America is currently threatened by the bud rot disease caused by *P. palmivora*. The disease has been reported in various Latin American countries on various oil palm cultivars and plantation stages (Martínez et al., 2010). Numerous bacterial and fungal pathogens, including various *Phytophthora* species, were previously isolated from multiple infected oil palm parts globally and suggested as causal agents of bud rot disease (Alvarez et al., 1999, as cited in Torres et al., 2016, p. 321; Faparusi, 1973; Richardson, 1995). Until recently, *P. palmivora* has been proven to be the causal agent of bud rot disease in oil palm trees in Colombia through Koch's postulates method, where 80% of inoculated young oil palms developed the exact typical bud rot symptoms (Drenth et al., 2013).

Despite being the world's largest oil palm producer, Southeast Asia has yet to report any bud rot cases related to *P. palmivora* infection reported in the region (Lee & Lum, 2004; Mohamed Azni et al., 2019). Sharples and Lambourne (1922) described the bud rot disease of oil palms in Malaysia with symptoms at the base of the youngest leaves, causing the collapse of the oil palm. Nevertheless, no affected tissue samples were collected. Later, in Malaysia, oil palm bud rot disease was linked to *Oryctes rhinoceros*, or lighting strikes (Bunting et al., 1934). Another study found that 5% of bud rot incidents in Malaysia were fatal cases (Turner & Bull, 1967). This situation is made more concerning by previous research indicating that Malaysian oil palm planting materials are susceptible to *P. palmivora* bud rot infection based on trials conducted in an oil-palm growing area in eastern Colombia (Navia et al., 2014). Another study found that Malaysian *P. palmivora* isolates (PP3 and PP7) are highly pathogenic to cocoa, durian, and rubber but not healthy Malaysian oil palm planting (*Dura* × *Pisifera*) materials. Even in flooded environments, a localized brown lesion on wounded and inoculated oil palm seedlings did not develop between 28 and 126 dpi (Mohamed Azni et al., 2019).

However, the epidemic that devastated the Colombian oil palm industry and the susceptibility of Malaysian oil palm planting materials has raised awareness in other oil-producing countries, especially in Southeast Asia. This endemic pathogen, *P. palmivora*, has caused various diseases in other crops, such as durian and rubber, in Malaysia and Indonesia. Without good management practices, this highly virulent pathogen may have the potential and easily attack oil palm plantations and spread the bud rot disease in Southeast Asia.

**Infection Mechanism in Oil Palm.** *Phytophthora palmivora* is the initiator that causes initial lesions and leads to tissue colonization by other fungi and bacteria that further decompose palm bud, leading to bud rot (Torres et al., 2016; Velez et al., 2008). The bud rot disease causes small brown lesions with a water-soaked edge at the base of the spear leaves, which quickly spread to the developing leaflets. When the lesion starts to develop on the edge of the leaflet, the symptoms worsen with the detachment of the middle lamella exhibiting a shot-hole appearance on the middle leaflet, the bite-like symptoms development, and the destruction of the interveinal tissue (Torres et al., 2016; Villa et al., 2013). Under high rainfall and high humidity conditions, sequential infection will aggressively affect the leaflets near the infected palm, causing more severe lesions. The infected palm's meristem and developing spears are destroyed in advanced stages. With these severe symptoms, no more young leaves will develop, and the bud will further deteriorate due to the invasion of pathogens and insects (Torres et al., 2016).

## Black Pepper (*Piper nigrum* L.)

Popularly known as the "King of Spices" or "Black Gold", black pepper (*Piper nigrum* L.) is one of the most essential spices used across the globe. Black pepper is primarily grown in Kerala, India, and has since been spread to other nations, predominantly in Southeast Asia (Hao et al., 2012). As of 2016, Vietnam is known as the world's greatest producer of black pepper, with a total production of 140,000 metric tonnes, followed by Indonesia (70,000 metric tonnes), India (48,500 metric tonnes), and Brazil (45,000 metric tonnes) (Hao et al., 2012; Ten, 2017, as cited in Takooree et al., 2019, p. S211). Apart from its immense importance in international trade and as a gourmet spice, black pepper is also beneficial for its diverse therapeutic purposes (Takooree et al., 2019).

*Phytophthora* foot rot is known as one of the most significant threats to the production of black pepper across the growing region worldwide. The disease was first reported in Indonesia in 1885 (Erwin & Ribeiro, 1996). The disease symptoms observed on infected black pepper in Sarawak are yellowing, defoliation, and collar rot. Due to limited knowledge of the morphological and molecular characterization of the *Phytophthora* sp. in the early stage of its discovery, it was difficult to identify the main causal agent of *Phytophthora* disease in black pepper. After examining several isolates from various hosts and geographical areas, Turner (1960) concluded that all the *Phytophthora* isolates from *Piper* in Southeast Asia belong to the same species and should be described as an atypical strain of *P. palmivora*. It is supported by a review that also recognized black pepper isolates as an atypical strain due to their unique morphological features not found in the MF1 strains (Waterhouse, 1974). However, a later study revealed that the pathogens isolated from black pepper in Thailand did not match the description of the atypical strain of *P. palmivora* or any of the *Phytophthora* sp. reported pathogenic to the black pepper.

The isolates have long pedicels on the caducous (deciduous) sporangia (Tsao & Tummakate, 1977). These findings further generate interest among researchers who have made extensive efforts and tried to compare the cocoa isolates of *P. palmivora* worldwide. Originally, there were two morphological forms of *P. palmivora* found in cocoa in West Africa based on their chromosome, which are 'S type' (small chromosomes [n = 9-12] at metaphase) and 'L type' (large chromosomes [n = 5-6] at metaphase). Later, the 'S' and 'L' types were redesignated and further classified into MF1, MF2, MF3, and MF4 based on the morphology characterization (Brasier & Griffin, 1979; Griffin, 1977). Most sporangia of MF1 had a rounded base, papillated and shed with a short, occluded stalk (pedicel) that was typically < 5 pm in length. The culture of MF1 on CA is stellate or radiate and has a smooth-combed appearance. The characteristic of MF2 isolates was similar to MF1, while MF3 was reclassified as *P. megakarya* (Brasier & Griffin, 1979; Griffin, 1977). In contrast, the MF4 Brazilian isolates have a papillated sporangia with long, non-occluded stalks (pedicel) and a sporangial base tapered to its connection with the pedicel giving a "sloping shoulders" appearance (Griffin, 1977). The black pepper isolates from Malaysia and Thailand showed similar characteristics to the Brazilian MF4 cocoa isolates, in which both have different characteristics from the typical *P. palmivora* strains. Therefore, Tsao and Tummakate (1977) believed that the *P. palmivora* isolates from black pepper should be classified as different species. Later, through taxonomic and genetic studies of the isolates, *P. palmivora* MF4 was reclassified as *Phytophthora capsici* (Alizadeh & Tsao, 1985). These species' reclassification was further confirmed through evolutionary analysis (Cooke et al., 2000). In a recent study, a pathogen isolated from infected black pepper in Sarawak displayed classic morphological characteristics of *P. capsici* by producing globose oogonia and paragynous antheridia, chlamydospore, and lemon-shaped sporangia with long pedicels (Farhana et al., 2013).

**Coffee (*Coffea arabica*)**

Coffee (*Coffea arabica*) is one of the most important commercial crops globally, cultivated in more than 80 countries, and contributes significantly to the livelihoods of smallholder farmers (Pham et al., 2019). Global coffee consumption was steadily growing and projected to grow by 3.3%, reaching 170.3 million 60-kg bags in 2021/2022 compared to 164.9 million 60-kg bags produced in the previous year (International Coffee Organization [ICO], 2022a). *Coffea arabica* and *Coffea canephora* (robusta) are the two most widely cultivated coffee species worldwide, which account for 60% and 40% of worldwide production, respectively (Leitão, 2019; Vega et al., 2020). Vietnam is known as the major coffee exporting country after Brazil, with 29,000 60-kg bags of coffee produced in 2020, followed by Indonesia (12,100 60-kg bags) and Thailand (500 60-kg bags) (ICO, 2022b).

Regarding *Phytophthora* root and stem rot disease, there is only one incident reported in 2001 that attacked a coffee orchard in Thailand, and it was said to be insignificant as the disease only causes 0.4% infestation (Sangchote et al., 2004). However, this incidence shows that coffee can be susceptible to *Phytophthora* infection. It can be concerning considering the widespread practice of intercropping among smallholder farmers. Intercropping practice helps to increase the diversity of an agricultural ecosystem and provide additional income in the years prior to the main crop production (Mousavi & Eskandari, 2011; O'Gara, Guest, et al., 2004). Together with langsat, longan, and papaya, coffee is among the crops planted for intercropping practice by Vietnamese farmers in durian orchards to provide shade and additional income (O'Gara, Guest, et al., 2004). A survey on a cocoa plantation in Nigeria also revealed that all farmers intercropped their cocoa plantation with various food crops, including coffee (Oladokun, 1990). As coffee is widely used for intercropping durian and cocoa, *P. palmivora* inoculum has a high probability of evolving and initiating a host jump from their primary hosts to coffee. Thus, extensive control measures must be taken to prevent the disease outbreak caused by *P. palmivora* in coffee.

## MANAGEMENT OF *PHYTOPHTHORA PALMIVORA*

*Phytophthora palmivora* has become a danger to important crops and has wreaked havoc on the global economy, so effective disease management techniques are required. The Malaysian industry is currently concerned about *P. palmivora*'s attacks on cacao and durian (Mohamed Azni et al., 2019). Most of the commercial cultivars of oil palm, cocoa, and durian are highly susceptible to diseases caused by *P. palmivora*. Therefore, disease management, such as cultural practices, disease-resistant germplasm, chemical control, and biocontrol, are studied and applied. Although many methods exist, effective disease control often combines several strategies (Drenth & Sendall, 2004).

### Cultural Control

*Phytophthora palmivora* thrives in a tropical and subtropical climate that favors the spread and multiplication of the pathogen (Martínez et al., 2010). Therefore, regulating water systems and irrigation is crucial to controlling *Phytophthora* diseases. The amount, frequency, and duration of plants exposed to water and water drainage must be seriously considered. Several suggested methods can be considered to control water drainage and prevent *Phytophthora* diseases. Farmers are urged to avoid growing plantations in forest regions, flood-prone places, and areas with poor drainage because of the possibility of water-assisted dispersal. The selection of fields with low *P. palmivora* concentration and a good drainage system is the first line of defense against this disease. Farmers may also grow their plants in a highly porous potting soil mix or plant their crops on raised beds (Pscheidt & Ocamb, 2022).

Fruit postharvest handling and storage are crucial to avoid contamination from phytopathogens, especially from an easily spread pathogen like *P. palmivora*. For instance, rinsing jackfruits with chlorinated water after sorting and grading them is a common phyto-sanitation measure practiced in India to remove foreign matter, latex, and field contamination to avoid fruit rot disease. Before fungicide application, phyto-sanitation was performed, which included removing decaying plant material, weeding, pruning, thinning, and shade reduction. A study in India proved that implementing such cultural practices as nutrient management, pruning, and field hygiene reduced cocoa black pod rot disease by 50% (Peter & Chandramohanan, 2014).

**Chemical Control**

Agrochemicals are widely used to increase yields and protect crops from pests and pathogens to protect crops from pests and pathogens. Chemical controls are intended to reduce pest and pathogen populations without harming crops (Brunner, 2014). Fungicide application is a conventional strategy for controlling phytopathogen diseases. Common fungicides to combat *P. palmivora* diseases include metalaxyl, mefenoxam, and phosphonates (Drenth & Guest, 2004; Torres-londono, 2016).

Metalaxyl (methyl-*N*-(2,6-dimethylphenyl-*N*-(2-methoxyacetyl)-DL-alaninate) is a well-known fungicide for controlling *P. palmivora* diseases. Metalaxyl inhibits fungal RNA synthesis (Drenth & Guest, 2004; Torres-londono, 2016). Metalaxyl can slowly penetrate through the leaf cuticle and into the xylem of a low lipophilic host (Phetkhajone et al., 2021). After penetration, metalaxyl accumulates and is transported upward by the transpiration stream. The study also concluded that metalaxyl foliar application at 4g/L is sufficient to control *P. palmivora* in two months. However, a lower concentration is suggested for soil drenching to treat the root and stem rot disease in durian trees (Phetkhajone et al., 2021). Protectant fungicide, mefenoxam, is a purified active isomer of metalaxyl that can affect mycelium growth and sporulation (Schwinn & Staub, 1995; Ware & Withacre, 2004). Furthermore, 30 g a.i./ha is the recommended rate for mefenoxam (Torres-londono, 2016). Durian farmers depend more on metalaxyl than other fungicides to control *Phytophthora* diseases. In Southern Thailand, the metalaxyl fungicide is used excessively, approximately 2-3 times per month, especially during the rainy season from May-October. Consequently, long-termed and excessive use of metalaxyl fungicide has resulted in the development of metalaxyl-resistant isolates with a 50% effective concentration ($EC_{50}$) higher than 100 mg/L (Kongtragoul et al., 2021). This issue will have a serious economic impact on the farmers as they are still applying the same metalaxyl-based fungicides but with higher dosage and greater frequency. The excessive fungicide application will further become hazardous to humans and the environment.

On the other hand, due to the high cost of metalaxyl-based fungicides and plant pathogens' resistance to metalaxyl, phosphorous acid has become another inexpensive alternative for oomycetes di management (Gómez-merino & Treio-Téllez, 2015). Phosphonates are proven to be highly effective against *Phytophthora* diseases in various crops. This inorganic compound acts synergistically with plant physiology to control the infection (Montiel et al., 2013). Both downward and upward phosphonate translocation are known as xylem-and phloem-translocated in the host (Ouimette & Coffey, 1990). They cause fungistasis and activate the host defense response by disrupting the pathogen's phosphorus metabolism (Drenth & Guest, 2004; Guest et al., 1995). Due to their phloem-translocated nature, phosphonates can be applied to any plant part and transported to other parts based on the source-sink relationship. Phosphonate fungicides in fosetyl-aluminum are widely used in the Philippines to overcome durian patch canker. Unlike metalaxyl, no phosphonate-resistant isolates of *Phytophthora* spp. have been reported after 20 years of use (Drenth & Guest, 2004).

There are multiple sites of action for these compounds (systemic, semi-systemic, or in contact with various compounds), and their efficiency varies depending on the application method, dosage, and time of year (Akrofi et al., 2003; Deberdt et al., 2008). Fungicides can be applied by drenching, foliar spray, stem canker paint, or trunk injection. However, each application has its downsides. For example, the foliar sprayed fungicide may not reach higher branches, allowing infected plant parts to remain on top and eventually infect the rest of the trees. Also, continuous rainfall may wash the fungicide off, which requires its frequent re-application. Due to soil degradation and leaching, soil drenching might also be ineffective (Drenth & Guest, 2004). Trunk injection application has proven to be a cost-effective alternative to overcome these problems, as it minimizes chemical waste while maximizing persistence (Darvas et al., 1984). For example, trunk injection of phosphite was proven to control severe root rot in avocado trees (*Phytophthora cinnamomi*) and trunk root rot in peach trees (*Phytophthora cactorum*) (De Boer et al., 1990). In a recent study, phosphonate application through trunk injection was proven effective against *P. palmivora* patch canker of durian in the Philippines, with a significant rise in yield at $US3.00 per tree/year (Montiel et al., 2013).

**Biocontrol**

In the intervening years, chemical fungicides have been widely used to control diseases. It has the most effective method to combat *Phytophthora* diseases (Sunpapao & Pornsuriya, 2014). However, the dramatic effect on pests and pathogens resulted in their overuse, which favored the development of fungicide-resistance pathogens. Consequently, higher chemical doses are introduced to protect crops and plants, raising public concerns regarding fungicide residue in food products, the

expensive chemical fungicides, and their impact on soil (Syed Ab Rahman et al., 2018). In addition, villamizar-Gallardo et al. (2017) expressed their concern about the deleterious effects of fungicides on the fly *Forcipomyia* sp., which pollinates cocoa flowers, affecting the ecological system.

Over the past 25 years, researchers and farmers have gained the popularity of biological control as a cost-effective and environmentally friendly alternative to reduce agrochemical use and plant diseases effectively. There is increasing evidence that beneficial microorganisms can promote plant growth and provide new strategies to combat pathogen diseases. These microorganisms act as biocontrol by inhibiting pathogen infection within the host directly (via mycoparasitism, antibiosis, and nutrient competition) or indirectly (via triggering resistance responses intrinsic to the host) (Mejía et al., 2008).

Numerous studies have shown that antagonistic microbial agents can develop and promote host resistance against *Phytophthora* spp. (Table 2) (Bailey et al., 2008; Hanada et al., 2008, 2009; Samuels et al., 2000). *Trichoderma* spp., *Pseudomonas* spp., and *Chaetomium* spp. are among well-studied genera that have attracted the attention of researchers and have proven to combat *P. palmivora* diseases.

*Trichoderma* spp. has been extensively used as an antagonistic fungal agent against several pathogens. These fungi's antagonism is based on faster metabolic rates, anti-microbial metabolites, and physiological conformation (Verma et al., 2007). In the interaction between *Trichoderma* spp. and *P. palmivora*, the most likely mode of action of *Trichoderma* spp. is parasitism and stimulation of the host's resistance reaction towards *P. palmivora* (Bailey et al., 2006; Harman et al., 2004). Recent research has examined the efficiency of *Trichoderma* spp. in controlling *P. palmivora* disease in durian, cocoa, and rubber (Table 2). *Trichoderma virens* (Tv) significantly reduces *P. palmivora* growth and disease development by producing antibiotic substances that inhibit the pathogen growth and act as mycoparasite, thus suggesting that it has multiple modes of action that contribute to its ability to suppress black pod rot and seedling blight of cocoa (Sriwati et al., 2015).

Instead of using a single crop management strategy, researchers have to combine *Trichoderma* spp. with potassium fertilizer to control cocoa black pod rot in Indonesia (Harni et al., 2020) (Table 2). Generally, potassium deficiency weakens plant resistance, allowing pathogens to penetrate (Damiri et al., 2011). Table 2 shows that *Trichoderma amazonicum* + 3g of potassium fertilizer (75.37%) suppresses leaf spots better than chemical fungicide (mancozeb) (70.86%). This ability of *T. amazonicum* to produce secondary metabolites that increase salicylic acid production may be the cause (Harni et al., 2019).

In recent years, more research has focused on antagonistic bacteria as a new environmentally friendly disease management strategy. *Pseudomonas* spp.

is popularly known to demonstrate high potential characteristics: the ability to produce antibiotics, plant growth promoters, hydrolytic enzymes, and hormones (Singh et al., 2013). Previously, various rhizosphere *Pseudomonas* spp. have been shown to control a range of plant pathogenic fungi and oomycetes (Acebo-Guerrero et al., 2015; Gravel et al., 2005; Miguélez-Sierra et al., 2019; Noori & Saud, 2012; Xu & Du, 2012). However, environmental conditions and competition for colonization in ecological niches can affect their potential and performance (Abraham et al., 2013). Due to these limitations, endophytic bacteria have recently gained popularity as a potential biocontrol agent against plant pathogens.

Several studies have been published on the antagonistic activity of endophytic *Pseudomonas aeruginosa* against *P. palmivora* infection on multiple hosts, especially in tropical countries (Kumar et al., 2005; Prakob et al., 2007; Siddiqui & Akhtar, 2007). It is supported by a study examining the effectiveness of 103 endophytic bacterial isolates isolated from healthy cocoa plants in seven Malaysian states against black pod rot disease caused by *P. palmivora* (Alsultan et al., 2019). Among all of the isolates, *P. aeruginosa* strain (AS1) that was isolated from the branch of a healthy cocoa plant in Perak, Malaysia, has been proven to have the highest inhibition rate against *P. palmivora* (Table 2) (Alsultan et al., 2019). The anti-fungal activity of *P. aeruginosa* is further supported by research demonstrating that *P. aeruginosa* produces several proteins capable of inhibiting the growth of *P. palmivora* with high inhibition activity (Table 2) (Sowanpreecha & Rerngsamran, 2018).

*Chaetomium* is one of the largest genera in the Chaetomiaceae family, and it lives primarily in soil and as endophytes in several plants via a symbiotic relationship (Fatima et al., 2016). *Chaetomium* species are effective biocontrol agents against multiple phytopathogens such as *Fusarium* spp., *Alternaria* spp., *Pythium* spp., and *Phytophthora* spp. Chaetomin mycotoxin was first isolated from *Chaetomium* spp. in 1944, leading to the discovery of a wide variety of bioactive compounds later on (Chen et al., 2015; Geiger et al., 1944). These compounds have been found to have significant biological activities such as anti-fungal, anti-inflammatory, cytotoxic, and enzyme inhibition (Li et al., 2016).

The effectiveness of *Chaetomium* application has been proven against *P. palmivora*, causing stem and root rot of durian in an epidemic area of infested field soil planted with durian in Thailand (Table 2). Furthermore, the 2-year field experiments proved that the application of *Chaetomium* spp. every four months, alongside good cultural practices, can effectively inhibit *Phytophthora* rot in durian and significantly reduce pathogen inoculum and disease incidence compared to metalaxyl treatment (Soytong, 2010). This finding is also supported by another research that has firstly reported the usage of nanoparticles (nanoparticles constructed from hexane crude extracts [nano-CCH]

Table 2
*Biocontrol application to control* Phytophthora palmivora *diseases*

| Biocontrol agent | Mechanism | Species | Host | Origin | Biocontrol effect | References |
|---|---|---|---|---|---|---|
| *Trichoderma* spp. | Antibiosis, mycoparasitism | *Trichoderma harzianum* | Rubber | Thailand | Controlled rubber leaf falls disease (66.22%) | Promwee et al. (2017) |
| | Antibiosis, mycoparasitism | *Trichoderma virens* | Cocoa | Indonesia | Produced antibiotic that inhibit pathogen growth and act as mycoparasite suppressing black pod rot and seedling blight of cocoa | Sriwati et al. (2015) |
| | Triggering host resistance response | *Trichoderma amazonicum* | Cocoa | Indonesia | Application with 3g potassium fertilizer have suppressed the number of leave spot, prolonged the incubation period, increased the growth of cocoa seedlings, and increased the lignin content in leaves (75.37%) | Harni et al. (2020) |
| *Pseudomonas* spp. | Antibiosis, competition | *Pseudomonas aeruginosa* (AS1) | Cocoa | Malaysia | Produce volatile metabolite and siderophores, causing the highest inhibition rate against *P. palmivora* growth (82.41%) | Alsultan et al. (2019) |
| *Chaetomium* spp. | Mycoparasitism, antibiosis, competition | *Chaetomium cupreum* (CC6)<br>*Chaetomium globosum* (CG7) | Durian | Thailand | Inhibit *P. palmivora* growth through bi-culture antagonistic with high antagonistic activity and greenhouse test (85.56%) | Soytong (2010) |
| | Antibiosis | *Chaetomium cupreum*: Nanoparticles; Nano-CCH and Nano-CCE particles | Durian | Thailand | Nano-CCH and nano-CCE (15 ppm) inhibit colony growth of *Phytophthora* spp. (86%) and inhibit sporangia formation by 96.42% and 97.32%, respectively | Thongkham et al. (2017) |
| | Antibiosis | *Chaetomium brasiliense*: Nano-CBH, Nano-CBE, and Nano-CBM particles | Durian | Thailand | Crude ethyl acetate from *C. brasiliense* significantly against *P. palmivora* ($ED_{50}$ value: 17.46 µg/ml)<br>Nanoparticles significantly inhibit colony growth (90%) and spore production (100%) | Tongon et al. (2018) |

and nanoparticles constructed from ethyl acetate crude extracts [nano-CCE]) from *Chaetomium cupreum* to inhibit *Phytophthora* spp. — causing durian root rot in Thailand (Table 2) (Thongkham et al., 2017).

**Transgenesis and Breeding**

Since the beginning of farming, plant pathogens have posed a significant challenge to food security despite all the control measures that have been introduced, such as agricultural practices, agrochemical use, and biological control. Over the past century, the development of disease control strategies has rekindled interest in transgenesis and host resistance breeding. Even though the earlier conventional breeding studies were primarily focused on major economic traits such as vigor and yield, the development of plants with disease resistance has drawn the attention of researchers over the years. Generally, there are three main components of resistance towards *Phytophthora* spp.: resistance to penetration, restriction of pathogen growth in the plant host, and the minimization of pathogen sporulation on the host.

The development of genetic resistance in cocoa trees has been proposed as the best alternative method for combating black pod rot disease (Susilo & Sari, 2014). Chang et al. (2020) screened a total of 50 potential cocoa genotypes available in Malaysia in which they tested their tolerance level against black pod rot. The *k*-means clustering method was used to assess disease severity based on the rate of lesion area development from 1 to 7 days post-inoculation and the proportion of pod area infected with black pod rot. All tested genotypes are categorized based on four tolerant levels: tolerant, moderately tolerant, moderately susceptible, and susceptible. Out of all the tested genotypes, 10 genotypes were classified in group I (tolerant) with six genotypes, namely MCBC 13, PBC 221, BAL 209, KKM 19, QH 1176, and KKM 22, which were found to have lower disease severity values as than the control tolerant genotype, PBC 123. Therefore, these genotypes can be recommended to farmers for use as planting material (Chang et al., 2020).

As for durian cultivation in Malaysia, the D24 clone was previously found to be more resistant to stem canker than D2 and D10. The interspecific hybrid crosses of D24 × D10 and D10 × D24 done by the Malaysian Agricultural Research and Development Institute (MARDI) have successfully produced MDUR 78 and MDUR 88 clones that were found to be more resistant than D24 (Sani et al., 2015). Both clones are proven to be resistant to *P. palmivora* stem canker and have the potential to produce high-quality fruits with higher yields. The fruits can also be stored for a long term and thus exported worldwide. Another durian breeding program in Thailand has found the $F_1$ hybrid of durian, IIICN-5-4-3-6, to be the most resistant against *P. palmivora* on stem and leaves (Somsri, 2014).

## CONCLUSION

In this review, the infection mechanism of *P. palmivora* in multiple crops, i.e., rubber, cocoa, durian, and oil palm, has been identified and described. The ability of *P. palmivora* to infect various plant parts in a wide range of hosts is a major threat to the future global spread and epidemic. Tropical and subtropical climates support the survival and propagation of *P. palmivora* inoculum due to the conducive environment, and the spores can easily be spread by wind and water. The pathogen infects rubber, cocoa, and durian trees in tropical regions. Among the reported cases are bud rot, pod rot, and stem canker severely affect oil palm, cocoa, and durian trees. Cultural control, chemical pesticide use, and biocontrol strategies have all been proven to control and minimize the spread of *P. palmivora* diseases. Recent studies have shown that *Trichoderma* spp. is the most effective antagonistic species against *P. palmivora*. Further research is necessary to identify hybrids resistant to *P. palmivora* diseases, as resistant hybrids are needed for disease prevention in plantations. Therefore, a better understanding of the *P. palmivora* infection mechanism in the main crops and early disease detection can reduce the risk of catastrophic pandemics.

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# Effect of Organic Waste Fertilizers on Growth and Development of Okra (*Abelmoschus esculentus*)

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## ABSTRACT

Okra (*Abelmoschus esculentus*) is an important vegetable crop grown with high demand and economic value. Thus, to improve the growth and development of okra, organic fertilizer can minimize inorganic fertilizer usage. The experiment was carried out in a greenhouse for 6 weeks to compare the growth rate of okra between a combination of organic waste fertilizers and NPK fertilizer and to determine the most suitable organic waste fertilizer combination with NPK fertilizer for the growth and development of okra. The experiment was laid out in a randomized complete block design (RCBD) with 4 replications consisting of 5 treatments, where T0: no fertilizer, T1: NPK 12:12:17:2 (20 g), T2: NPK 12:12:17:2 (10 g) + vermicompost (25 g), T3: NPK 12:12:17:2 (10 g) + biochar (25 g), T4: NPK 12:12:17:2 (10 g) + chicken manure (25 g), respectively. Parameters assessed were plant height, number of leaves, chlorophyll content, number of fruits, fresh and dry weight, and soil pH. Results indicated that the growth and development of okra were significantly the lowest in T0 and T1 while the highest in T4. Okra in T4 showed the best performance by achieving the highest value for all parameters assessed after 6 weeks of planting. It can be deduced that NPK 12:12:17:2 (10 g) + chicken manure (25 g) might be the most suitable fertilizer combination to promote the higher growth of okra while reducing the dependency on inorganic compound fertilizers.

*Keywords*: Biochar, chicken manure, NPK 12:12:17:2, okra, organic waste fertilizer, vermicompost

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## INTRODUCTION

In many developed countries, the vegetable crop has been the most important food resource for human livelihoods. Moreover, vegetable farming has become important

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in fulfilling the world's population growth rate requirement since it has become a vital food for nutrient supply. Okra (*Abelmoschus esculentus*) is an important and profitable vegetable crop grown from the Malvaceae family and cultivated in many countries, including Asia, Africa, southern Europe, America, and the Indo-Pak subcontinent (Kumar et al., 2013). Production of okra in the world is estimated at 6 million tons/year, and the production has risen over the years, with India estimated at 67.1% of the total world production, followed by Nigeria being 15.4% and Sudan at 9.3% (Sorapong, 2012).

Okra, or ladies' finger or *Bendi* in Malaysia, is popular due to its healthy aspects, such as high fiber, antioxidants, vitamin C, minerals, potassium, and calcium. It is also important in tropical and subtropical countries as a medicinal plant for plasma replacement (Kumar et al., 2013; Sorapong, 2012). Okra is popular due to its short production time and easy growing, and it is a multipurpose plant for its numerous uses for its fresh leaves, flowers, buds, pods, stems, and seeds. Furthermore, okra's potential for medicinal value has been recognized (Gemede, 2015). Okra mucilage can be used as a blood volume expander or plasma replacement for medical purposes. The okra's mucilage binds the cholesterol and toxins in bile acid will be excreted by the liver, and mostly the whole part of the okra is fit to eat and used for food (Gemede, 2015; Maramag, 2013).

The fertilizer requirement is important at early growth to increase okra's productivity and quality. Currently, chemical fertilizer such as NPK (nitrogen, phosphorus, and potassium) is widely used in agriculture, including in vegetable crop planting, as it results in high productivity in a short time. However, it is quite expensive and results in nutrient imbalance and soil acidity (Akande et al., 2010). Applying organic manure has shown a positive response by increasing soil microbes and plant health. Manures are frequently applied at a higher rate than inorganics. High rates have residual impacts on crop development and production. In many countries, combining organic and inorganic fertilizers may be helpful and relative to applying each material separately. As such, the combination usage of powdered rock phosphate and chicken manure considerably boosted the growth and development of okra (Akande et al., 2004). Hence, mixing compost, manure, and biochar with inorganic fertilizer could enhance plant growth. Therefore, the organic application may produce eco-friendly and sustainable okra farming. The combination of different organic waste fertilizers may ameliorate the high dependency on chemical usage while reducing the cost of inorganic fertilizers and improving the growth development and productivity of okra. Apart from improving crop output, the approach has a better residual effect than using organic or inorganic fertilizer alone (Akande et al., 2010).

Therefore, the objectives of this study were to compare the growth rate of *A. esculentus* between the combination of organic waste fertilizer and NPK fertilizer as well as to determine the most suitable

organic waste fertilizer combination with NPK fertilizer for the growth and development of *A. esculentus*. Comparing the different types of organic fertilizer can determine which organic waste fertilizer combinations have high growth rates and are most suitable as an alternative to reduce the dependency on inorganic fertilizer usage. This research also focuses on reducing the use of inorganic fertilizer in crops that negatively impact soil, the environment, and human health. Intensive use of inorganic fertilizer can be reduced by mixing both inorganic and organic fertilizers.

## MATERIALS AND METHODS

### Experimental Materials

This experiment was conducted in a greenhouse of Universiti Teknologi MARA (UiTM) Jasin Campus, Melaka, Malaysia (2°13'44.0" N 102°27'30.7" E). The experiment was held for 2 months, from November 2021 to December 2021. Since this experiment was done in the greenhouse, hence the microclimate in the greenhouse was as follows: temperature of 32 ºC, sunshine or more than 5.5 hours/day with more than 17 MJ per sq m per day, relative humidity of 80%, and mean wind speed of 10m/sec.

The experiment was conducted using certified okra seed from Leckat Brand (Malaysia) of F1 Hybrid Indian Ocean 535. Before planting, okra seeds were soaked overnight in a container filled with water to hasten the germination rate by softening the outer layer of okra seed. The planting of the okra seeds involved a transplanting method where the seeds were sown on germination trays filled with peat moss as planting medium during the initial stage. After 7 to 10 days, the seedlings started emerging with at least one true leaf where they were ready for transplant into a large polybag of 10 kg of topsoil (14" × 14").

The topsoil used in this study was a clay loam of Malacca series (Typic Hapludox) from a cultivated field in the Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA Agricultural Farm at Merlimau, Malacca. The soils were taken at 0–15 cm depth. Soil pH was determined on a 1:2.5 soil: distilled water suspension using a glass electrode (Peech, 1965). Soil texture was determined using the hydrometer method (Tan, 2005). Soil organic matter (SOM) content was determined by the loss-on-ignition method after placing samples in a muffle furnace at 300–550 °C for five hours (American Society for Testing and Materials [ASTM], 1988). The percentage of organic carbon is assumed to be 58% of the organic matter content. Bulk samples (undisturbed) were also collected for bulk density determination using stainless steel rings (diameter 5.2 cm, height 6.0 cm).

### Treatment Application

The organic waste fertilizers used were chicken manure, vermicompost, and biochar, while for inorganic fertilizer, NPK (Mg) 12:12:17:2 (Twin Arrow Fertilizer, Malaysia) was used. The number of fertilizers applied to each polybag was about 20 g of NPK 12:12:17:2 (T1), as recommended by the Department of Agriculture (DOA) (1997).

For other treatments, NPK 12:12:17:2 was integrated with organic waste fertilizers (Dsyira Enterprise, Malaysia), which were 25 g of vermicompost (T2), 25 g of biochar (T3), and 25 g of chicken manure (T4), respectively as shown in Table 1. Previous research referred to the rate of organic waste fertilizer use (Premsekhar & Rajashree, 2009; Sarma & Gogoi, 2015).

The organic waste fertilizers were applied into the soil one week before transplanting as recommended by DOA (1997), as organic amendments need to undergo decomposition before being able to release nutrients (N, P, K, and Mg) required by the plants (Chandini et al., 2019), where the organic waste fertilizers were mixed with soil to avoid from settling in one spot and ensuring uniform application and nutrient release as to allow the microbial activity to work and to improve the soil properties (Uka et al., 2013). Alternatively, the application for NPK 12:12:17:2 was conducted at week 2 and week 6 after transplanting that was applied 6-7cm away from the plant's collar to prevent the plant from scorching (DOA, 1997). Watering was done manually twice per day (1 liter/polybag). This study involved 5 treatments with 4 replications using a randomized complete block design (RCBD) (Table 1).

Table 1
*Treatments used in this experiment*

| Treatment | Description |
|---|---|
| T0 | No fertilizer |
| T1 | NPK 12: 12: 17:2 (20 g) |
| T2 | NPK 12: 12: 17:2 (10 g) + vermicompost (25 g) |
| T3 | NPK 12: 12: 17:2 (10 g) + biochar (25 g) |
| T4 | NPK 12: 12: 17:2 (10 g) + chicken manure (25 g) |

**Collection of Data**

Okra was harvested about 2 months after sowing. In this study, the parameters for the growth and development of okra that were measured to determine the effect of the treatments were plant height, number of leaves, chlorophyll content per leaf, number of fruits, soil pH, as well as fresh and dry weight of plants. The plant height was measured from the surface of mineral soil up to the highest tip of the plant using a measuring tape. The number of leaves per plant was measured by counting the leaves after the true leave emerged. The chlorophyll content in leaves was measured using Soil Plant Analysis Development, SPAD-502 meter (Konica Minolta, Japan). The meter was calibrated for around 15 minutes before taking readings correctly. Soil pH measurement was using a pH meter at a 1:2.5 soil-to-solution ratio (Enio et al., 2021). All the measurements stated above were recorded and collected every 2 weeks. In addition, a number of fruits were collected and recorded when the plant started to develop fruits. Finally, the fresh weight of the plant was measured by using the electronic balance in the laboratory right after harvesting the plant. In contrast, the dry weight of plant shoot and root was measured by using electronic balance after harvesting and underwent a drying process in the drying oven at approximately 60 °C for 5-7 days after a constant weight was achieved.

### Statistical Analysis

All the data was collected and recorded in the statistical package for social science (SPSS) (version 21) using analysis of variance (ANOVA) software to determine if there are any significant differences between the treatments. The mean data of the treatments were compared using Tukey's test. The significant difference was considered at $p < 0.05$.

## RESULTS AND DISCUSSION

### Soil Physical and Chemical Characteristics

The selected chemical properties of the soil, as shown in Table 2, were typical of Malacca Series and were consistent with those reported by Paramananthan (2000). Therefore, the pH and organic matter content were considered low, typical for such a series. According to Paramananthan (2000), soils of the Malacca series are lateritic soils, which contain high iron and aluminum while low in cation exchange capacity (CEC) and water holding capacity, thus explaining the low pH and organic matter content.

Table 2
*Selected physical and chemical characteristics of topsoil used in this study*

| Characteristics | Values |
|---|---|
| pH | 4.2 |
| Sand (%) | 35 |
| Silt (%) | 38 |
| Clay (%) | 33 |
| Organic matter (%) | 0.74 |
| Organic carbon (%) | 0.43 |
| Bulk density (g $cm^{-3}$) | 1.4 |

### Effect of Treatments on Plant Height in *Abelmoschus esculentus*

Figure 1 shows the effect of different treatments on the okra plant height (*Abelmoschus esculentus*) during 8 weeks of growth. During the initial week (week 2), the plant height in each treatment showed no significant differences. However, the plant started showing response to the treatments from week 6 onwards as T4 (NPK 12:12:17:2 10 g + chicken manure 25 g) showed the highest plant height (43.85 cm) significantly at week 8, followed by T3 (NPK 12: 12: 17: 2 10 g + biochar 25 g) with 34.65 cm in height, T2 at 25.83 cm in height while the least was found in T1 (NPK fertilizer alone) with only 19.18 cm in height at 8 weeks of plant growth after transplanting.

From week 4 to week 8, treatments added with organic waste fertilizers resulted in significant plant height ($p < 0.05$). It could be attributed to the organic wastes (vermicompost, biochar, and chicken dung) ability to improve soil fertility as a result of decomposition, and these waste materials may have released both macro and micronutrients necessary for plant growth while also improving the soil's physicochemical properties (Tiamiyu et al., 2012), thus justifying the increase in plant height. It can also be observed from Figure 1 that okra treated with poultry manure in T4 performed better than other organic fertilizers implying that decomposition in chicken manure might be more rapid due to its lower C: N ratio (Adekiya et al., 2020) and thus nutrients in such manure

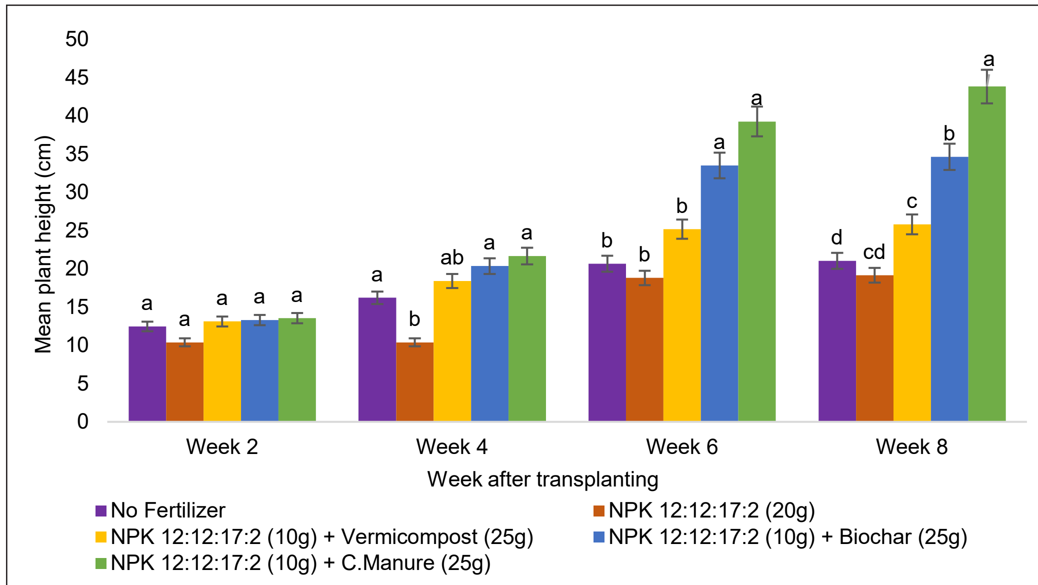


*Figure 1*. Effect of treatments on plant height of *Abelmoschus esculentus* during 8 weeks of growth
*Note*. Small letters display mean separation between treatments for a particular week using Tukey's test at a 5% level. Different letters are significantly different between treatments applied for the particular week.

was more readily available and in the optimal amount for root to absorb easily, thus explaining accelerated performance in plant height (Tiamiyu et al., 2012). Such observation agreed with the findings by Adekiya et al. (2020), Ajari et al. (2004), and Olaniyi et al. (2010) that poultry manure increased the height of okra relative to other amendments. Therefore, the superior N supply by poultry manure during okra cropping in this experiment may be the reason for the better growth and yield of okra in plots with chicken manure.

## Effect of Treatments on Number of Leaves in *Abelmoschus esculentus*

The effect of treatments on the number of leaves in okra was similar to the result in plant height, whereby the response towards the treatments was more prominent from week 6 onwards, as shown in Figure 2. Therefore, it can be observed that, generally, there were no significant differences between all treatments during weeks 2 and 4. However, at week 6, the number of leaves showed significant differences between treatments applied where T4 was found to contain the highest number of leaves (8.5 leaves), followed by T3 (6 leaves) and T2 (4 leaves), while the least number of leaves was found in T1 with only 2 leaves.

The number of leaves in week 6 for all treatments was higher compared to week 8 due to the leaves started to fall off. The increase in the number of leaves per plant caused by organic fertilizer treatment emphasized the necessity of organic fertilizer during plant vegetative growth (Tiamiyu et al., 2012). Majanbu et al. (1986) found that N and K are the most

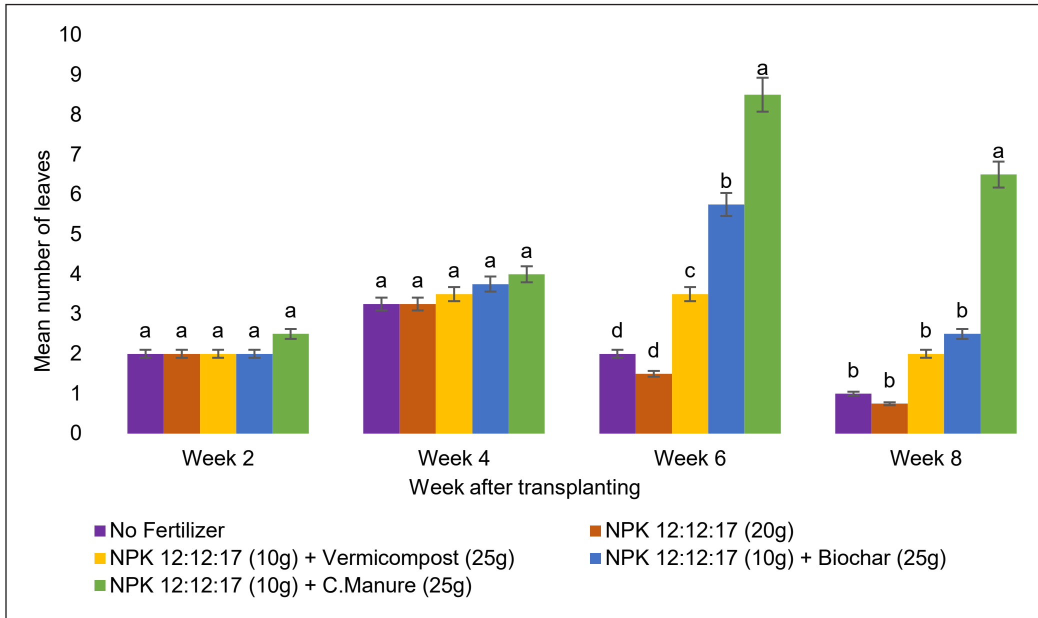


*Figure 2*. Effect of treatments on the number of leaves in okra (*Abelmoschus esculentus*) during 8 weeks of growth

*Note*. Small letters display mean separation between treatments for a particular week using Tukey's test at a 5% level. Different letters are significantly different between treatments applied for the particular week.

important macronutrients that okra required for proper growth and pod production and that chicken manure is known to have high concentrations of N and P as well as low carbon: nitrogen (C: N) ratio. Also, nitrogen is well known to be the major constituent of chlorophyll, protein, amino acids, various enzymes, nucleic acids, and many other compounds in the cell of plants (Agbede, 2009). Hence, the relatively high nitrogen concentration of chicken manure promotes crop vegetative growth and thus increases the number of leaves. According to Uka et al. (2013), organic fertilizer had a good influence on vegetative development. Moreover, okra grown on chicken manure outperformed other organic soil amendments and NPK fertilizers in terms of growth and yield. These characteristics of poultry manure result in rapid mineralization and early nutrient release in a short gestation crop like okra, increasing morphological growth such as the number of leaves (Adekiya et al., 2020).

### Effect of Treatments on Chlorophyll Content in *Abelmoschus esculentus*

Figure 3 displays the mean for chlorophyll content (μg $cm^{-2}$) as affected by the different treatment applications starting from week 4 to week 8. In general, it can be observed that throughout the weeks, treatment with organic waste fertilizer resulted in significant chlorophyll content ($p < 0.05$), where chlorophyll content was significantly the highest in T4 (NPK 12:12:17:2 10 g + chicken manure 25 g), which was 78.68 μg $cm^{-2}$, followed by T3 (NPK 12:12:17:2 10

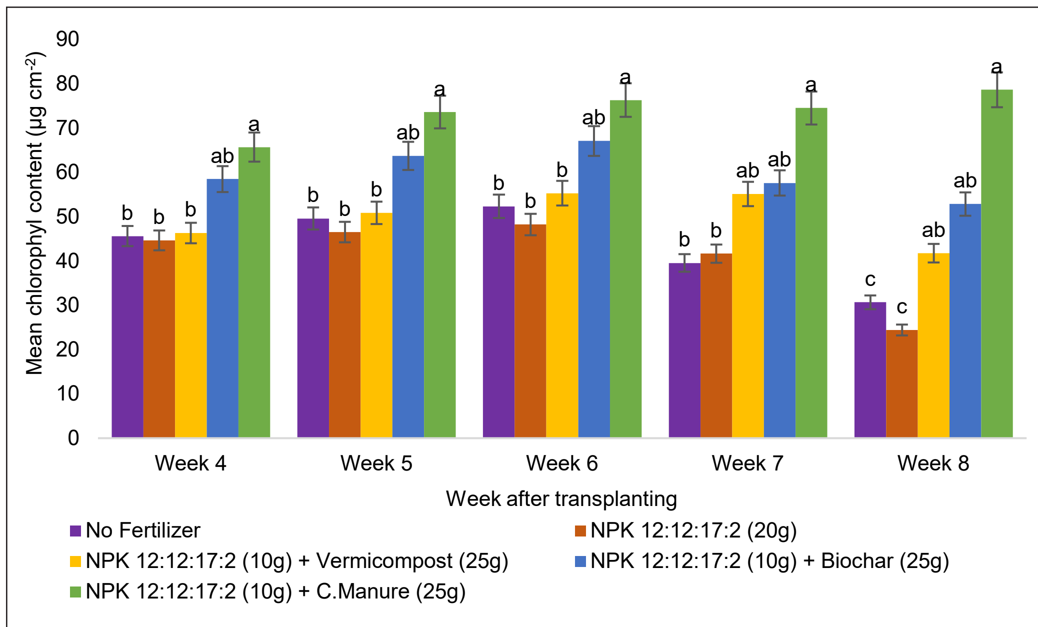


*Figure 3*. Effect of treatments on chlorophyll content in okra (*Abelmoschus esculentus*) during 8 weeks of growth

*Note*. Small letters display mean separation between treatments for a particular week using Tukey's test at a 5% level. Different letters are significantly different between treatments applied for the particular week.

g + biochar 25 g) containing 52.88 µg $cm^{-2}$, T2 (NPK 12:12:17:2 10 g + vermicompost 25 g) recorded about 41.75 µg $cm^{-2}$, T0 (no fertilizer) which was 30.7 µg $cm^{-2}$, and the lowest mean of chlorophyll content was observed in T1 (NPK 12:12:17:2 20 g), which recorded about 24.45 µg $cm^{-2}$. The use of an organic source of nutrients may have greatly increased the chlorophyll content in the leaves (Premsekhar & Rajashree, 2009). Also, since the number of leaves was greatly affected by the application of treatments, the amount of total chlorophyll content in the plant was also increased as the total number of leaves increased.

One of the most important biomolecules is chlorophyll, critical for photosynthesis, allowing plants to get energy by absorbing sunlight (Khandaker et al., 2017). The increased use of organic manures, which contains significant amounts of magnesium, may have benefited chlorophyll synthesis, increasing the rate of photosynthesis (Premsekhar & Rajashree, 2009). In addition, the magnesium in organic fertilizer is involved in chlorophyll production, which will increase the rate of photosynthesis. Furthermore, the application of organic manures would have helped the metabolic activity of plants by supplying essential micronutrients during the early stages of robust growth (Premsekhar & Rajashree, 2009). According to Khandaker et al. (2017), the amount of chlorophyll varies depending on the factors of edaphic and climatic conditions, such as water and light stress and fertilizer as well as depending on the phase of the vegetation cycle.

### Effect of Treatments on Number of Fruit in *Abelmoschus esculentus*

Figure 4 displays the mean number of fruits affected by treatment applications for weeks 5, 6, 7, and 8. The application of chicken manure with NPK fertilizer (T4) significantly showed the highest number of fruits in *A. esculentus,* which was 4, followed by T3 (NPK 12:12:17:2 10 g + biochar 25 g), which was 2.5, T2 (NPK 12:12:17:2 10 g + vermicompost 25 g) which is 1.5. Conversely, the lowest number of fruits was in T0 (No fertilizer) and T1 (NPK 12:12:17:2 20 g), which was 1.

Animal manures, when handled efficiently, promote long-term agricultural productivity by immobilizing nutrients that are prone to leach. Nutrients in manure are released more slowly and are kept in the soil for a longer period, resulting in longer residual effects, increased root development, and higher crop yields (Akande et al., 2010). Besides that, the response of high yield due to organic fertilizer is attributed to the improvements in soil's physical and biological qualities, which resulted in increased nutrient availability and improved crop growth and production (Premsekhar & Rajashree, 2009).

### Effect of Treatments on Fresh and Dry Weight in *Abelmoschus esculentus*

Figure 5 displays the mean fresh and dry weight (g) as affected by treatment applications, where it can be observed that no significant differences were observed between T0, T1, T2, and T3. It was only in T4 where the weight was significantly different from other treatments and showed the highest mean of fresh and dry weight, 59.29 g and 9.08 g, respectively. The increase in fresh weight of okra with the

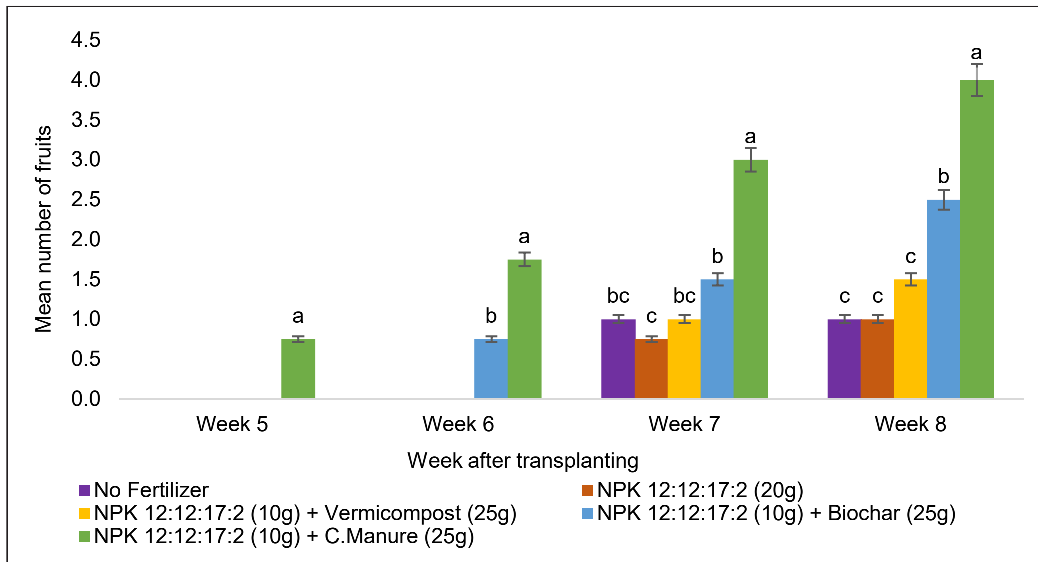


*Figure 4*. Effect of treatments on a number of fruits in okra (*Abelmoschus esculentus*) during 8 weeks of growth
*Note*. Small letters display mean separation between treatments for a particular week using Tukey's test at a 5% level. Different letters are significantly different between treatments applied for the particular week.

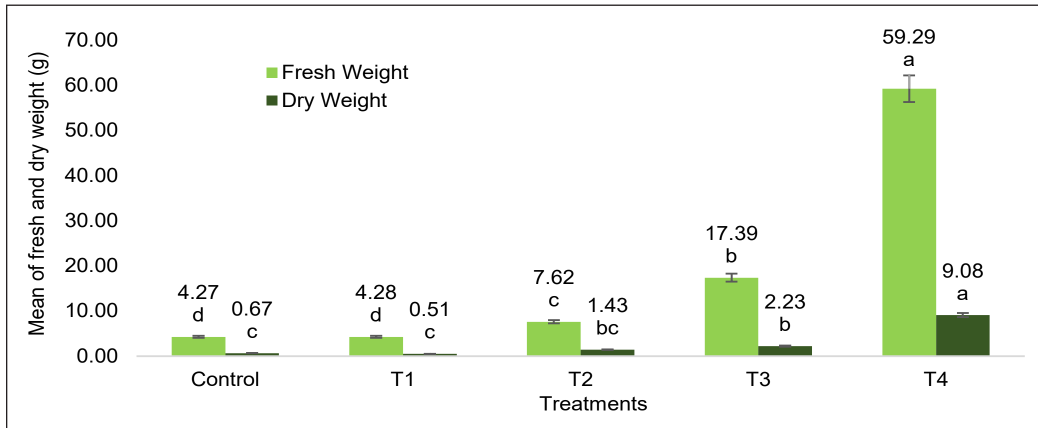


*Figure 5*. Effect of treatments on fresh and dry weight (g) in okra (*Abelmoschus esculentus*) at the end of 8 weeks of growth

*Note*. Small letters display mean separation between treatments within a particular type of weight measured (fresh or dry) using Tukey's test at a 5% level. Different letters are significantly different between treatments applied for the particular week.

application of organic manure could be related to the efficient effect of solubilization of the released plant nutrients, resulting in improved soil nutrients and a water-holding capacity (Tiamiyu et al., 2012). The results corroborated the finding of Premsekhar and Rajashree (2009) in Okra (*A. esculentus*), which indicated that increased yield response of crops following organic manure application could be attributed to improved physical and biological properties of the soil, resulting in an adequate supply of nutrients to the plants (Premsekhar & Rajashree, 2009). When nutrients are available in the appropriate quantities, photosynthetic activity will increase, resulting in an increase in light interception, dry matter formation, accumulation, and partitioning (Uka et al., 2013). In addition, the dry matter weight of the plant will rise as a result of greater root development, which will promote water translocation and nutrient uptake to plant parts (Razak et al., 2017).

## Effect of Treatments on Soil pH

It can be observed in Figure 6 that during the initial week (week 1) of planting, the soil pH was the same regardless of treatments which were quite acidic (pH 4.19). However, from week 3 to week 8, treatment with organic waste fertilizer started to display a significant increase in pH. However, okra applied with T4 (NPK 12:12:17:2 10 g + chicken manure 25 g) significantly showed the highest soil pH value from week 3 to week 8, which was about pH 5.2, followed by T3 (NPK 12:12:17:2 10 g + biochar 25 g) which was pH 5.03, T2 (NPK 12:12:17:2 10 g + vermicompost 25 g) recorded at pH 5.02, T1 (NPK 12:12:17:2 20 g) resulted at pH 4.79 and the lowest mean of soil pH value was T0 (No fertilizer) which was at pH 4.47. Such increase in pH after treatment applications were possibly attributed to ion exchange reactions which occur when terminal hydroxide ($OH^-$) of aluminum

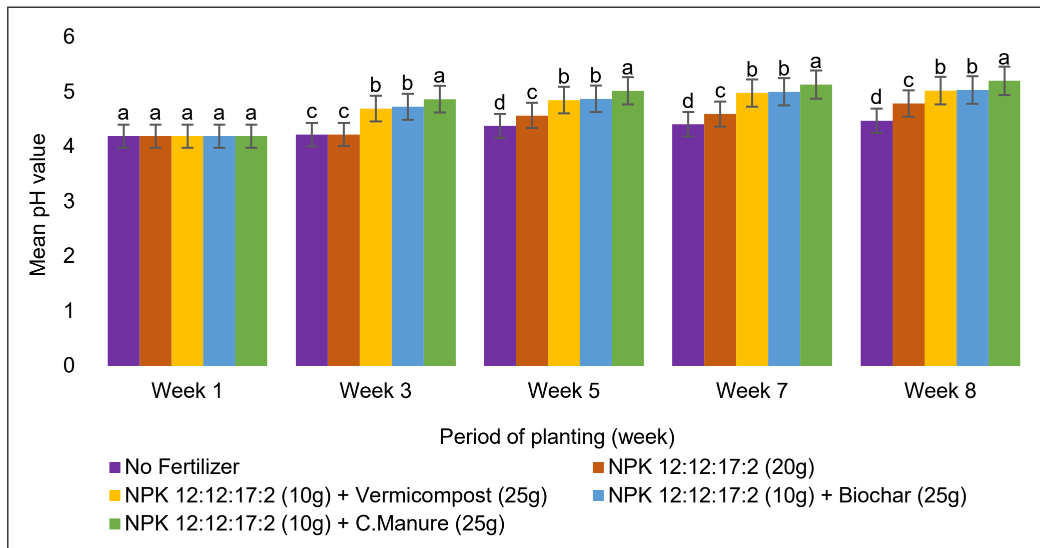


*Figure 6*. Effect of treatments on soil pH planted with okra (*Abelmoschus esculentus*) during week 1 (before transplanting) to week 8 of growth

*Note*. Small letters display mean separation between treatments for a particular week using Tukey's test at a 5% level. Different letters are significantly different between treatments applied for the particular week.

(Al) or ferrous ion ($Fe^{2+}$) hydroxyl oxides are replaced by organic anions, which are decomposition products of the manure such as malate, citrate, and tartrate (Bessho & Bell, 1992; Hue & Amiens, 1989; Pocknee & Summer, 1997; Van et al., 1996).

Another explanation was the presence of basic cations in poultry manure, such as calcium (Ca) and magnesium (Mg). Nätscher and Schwertmann (1991) reported that such basic cations are released upon microbial decarboxylation during decomposition. Organic manures promote soil fertility by stimulating microbial activity and biomass in the soil, increasing the physical and biological properties of the soil (Akande et al., 2010). The addition of organic materials was said to increase soil pH, and this result agreed with the findings by Akande et al. (2004) and Duruigbo et al. (2007) that the application of organic materials will ameliorate crop production in slightly acidic tropical soil. Hence, the application of organic fertilizer can act in 2 ways, providing nutrients for plants and increasing soil pH as it acts as a liming material.

Overall, this experiment was able to successfully substantiate that reducing the dependency on the amount of NPK fertilizer to only half of the amount applied and by adding organic waste fertilizer not only able to produce yield comparable to having applied the full amount of NPK fertilizer that is required but also produced a yield that is significantly much higher than applying NPK fertilizer alone. Hence, since the okra treated with the combination of NPK fertilizer and chicken manure showed the highest growth and development compared to other organic waste fertilizers; therefore, such practice in the production of vegetables like okra should be encouraged.

### Recommendations

It is highly recommended for farmers to apply chemical fertilizers according to the standard timing of application (week 2 and week 6 after transplanting) but with half of the recommended dosage to reduce the dependency on chemical fertilizers while also thoroughly mixing chicken manure with the planting medium at a rate of 25 g/10 kg of soil. Not only will this reduce the amount of wastage from poultry farming, but it will also reduce the cost of buying chemical fertilizer while recycling and performing agricultural activities in an eco-friendly approach.

## CONCLUSION

The application of organic waste fertilizers, such as chicken manure, biochar, and vermicompost, had a significant effect on the growth and yield of okra (*Abelmoschus esculentus*), particularly plant height, the number of leaves and fruits, chlorophyll content, fresh and dry weight, and soil pH compared to the application of NPK fertilizer alone. Okra treated with the combination of 10 g of NPK fertilizer (12:12:17:2) with 25 g of chicken manure showed the highest growth and development compared to other organic waste fertilizers.

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*Review Article*

# Hormone Application for Artificial Breeding Towards Sustainable Aquaculture – A Review

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## ABSTRACT

Aquaculture has been the fastest-growing area of worldwide food production and is becoming a vital component of the global economy to feed the rising world population. Hence, directed toward continuing the current level of per head consumption, comprehensive aquaculture production needs to attain eighty million tonnes by 2050. However, some cultured marine fish species, such as salmonids, striped bass, and gilthead seabream, as well as freshwater fish, such as captive Mediterranean amberjack populations (*Seriola dumerili*) and Mekong River giant catfish (*Pangasianodon gigas*), exhibit reproductive dysfunction, especially in female brood stock when reared in captivity. Captive females face complications with unsynchronised ovulation, fail to undergo final oocyte maturation (FOM), and no longer spawn due to a lack of luteinising hormone (LH). Thus, artificial breeding has been widely used in aquaculture practices to increase cultured fish production. Farmer has extensively applied commercial hormones such as human chorionic gonadotropin (hCG), Ovaprim, Ovatide, and Ovaplant, through injection and implantation of hormones to stimulate breeding in many farmed fish species. However, artificial breeding is still in its development phase, and some methods are still unable to induce spawning in certain fish species. Different methods, doses, and delivery systems of artificial hormones could improve the efficiency and effectiveness of artificial breeding. This paper discusses the current research on artificial breeding in various fish species as well as new

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approaches or techniques to be applied in the area to regulate the reproductive process in captive fish for sustainable aquaculture.

*Keywords*: Artificial breeding, artificial hormones, fish reproduction, synthetic hormones, sustainable aquaculture

## INTRODUCTION

For the past three decades, aquaculture has been the world's fastest-growing food production subsector, producing more than half of all the fish consumed directly by humans (Filipski & Belton, 2018). This industry contributes to human nutrition in ways that are essential for human health through its essential proteins and micronutrients, including calcium, vitamin A, vitamin B12, and omega-3 fatty acids (Carbone & Faggio, 2016; Jayasankar, 2018). With the continuous growth of the world's population, food and feed supply should rise by 70% by 2050 (de Bruijn et al., 2017). Consequently, the aquaculture supply is expanding due to the rising global demand for fish (Karim et al., 2020). Asian countries contribute approximately 89% to the worldwide aquaculture output, with China dominating (61.5% of the global aquaculture output), followed by India, Indonesia, Vietnam, Bangladesh, Egypt, Norway, Chile, Myanmar, and Thailand (Ahmed & Thompson, 2019).

Fish culture is a significant industry, cultivating various types of marine and freshwater fish worldwide. Fish are often raised in confined areas, such as ponds or net cages, with efforts to maximise the production per unit area (Mehana et al., 2015). Domestication is the capacity of fish to regulate reproductive processes in captivity and obtain good quality seeds for commercialisation (Mylonas et al., 2010; Passini et al., 2019). Most captivity-raised fish experienced some form of reproductive failure. These dysfunctions are most likely due to a mix of captivity-induced stress and differences in social and environmental factors between wild and farmed fish. This combination of adaptations initiates the endocrine mechanisms that lead to final oocyte maturation (FOM) and ovulation (Zohar & Mylonas, 2001). Previous research linked impaired hormonal regulation at the hypothalamus-pituitary-gonadal (HPG) axis to poor reproductive success due to the lack of reproductive control, low sex hormone levels, slow or delayed gametogenesis, and small egg size (Milla et al., 2020). Females in captivity frequently failed to complete FOM, ovulation, and spawning, while males experienced decreases in sperm quantity or quality (Mylonas et al., 2007; Zohar & Mylonas, 2001). These problems have slowed aquaculture production, resulting in a shortage of seeds. Thus, artificial breeding is an excellent method for restocking wild populations and endangered species.

The HPG axis in fish regulates gametogenesis and final maturation (Mylonas et al., 2010). It begins with environmental stimuli (water rise, temperature, feeding, rainfall, and photoperiod) that induce the brain's release of gonadotropin-releasing hormones (GnRHs) (Figure 1) (Fakriadis et al., 2020). GnRHs stimulate gonadotropin

hormones (GTHs) in the anterior pituitary gland, namely, luteinising hormone (LH) and follicle-stimulating hormone (FSH). FSH regulates vitellogenesis and spermatogenesis in endocrine systems, while LH regulates FOM and spermiation (Mosha, 2018). Fish in captivity often lack environmental cues. Therefore, various hormones that promote fish reproduction should be applied to reduce reproductive failure in captivity (Mosha, 2018; Mylonas et al., 2010). In addition, this approach guarantees the availability of fish seeds throughout the year (Ochokwu et al., 2015). Given that many tropical fish species only reproduce once a year (Ochokwu et al., 2015), artificial breeding may also be one of the best solutions to meet the demand and could reduce the fish seeds that rely on the wild.

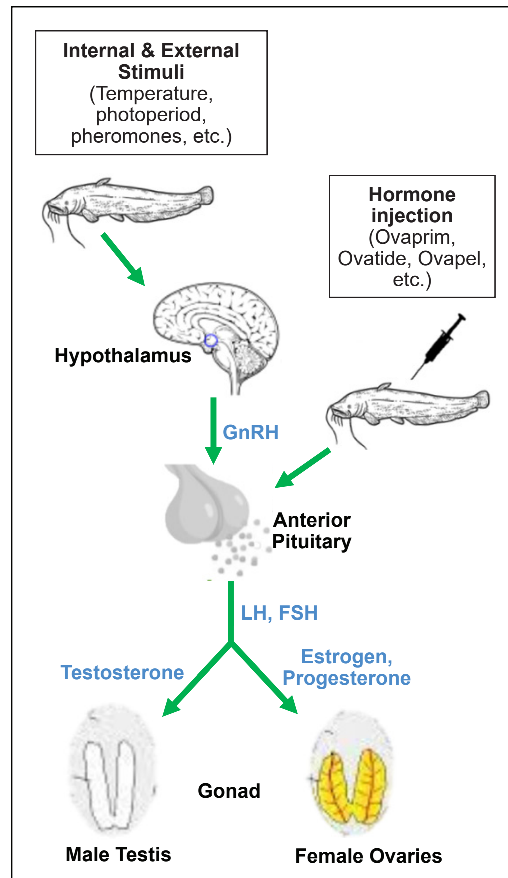


*Figure 1*. Schematic diagram of hypothalamus-pituitary-gonad (HPG) axis

*Note*. GnRH = Gonadotropin-hormone releasing hormone; LH = Luteinising hormone; FSH = Follicle stimulating hormone

## The Development of Hormones for Artificial Breeding

The advancement of induced spawning techniques has enabled farmers to commercially breed valuable fish in captivity (Marimuthu, 2019). The administration of exogenous hormones, such as carp pituitary extract (CPE), human chorionic gonadotropin (hCG), luteinising hormone-releasing hormone analogue (LHRHa), and salmon gonadotropin-releasing hormone analogue (sGnRHa), is an alternative method of imitating environmental and hormonal factors to promote ovulation and egg maturation in fish for efficient seed production (Su et al., 2013). Several commercial ready-to-use synthetic hormones contained gonadotropin-releasing hormone analogue (GnRHa) and dopamine antagonists (domperidone, DOM), such as Ovaprim, Ovopel, Ovatide, Ovupin-L, Dagin, Aquaspawn, and Ovulin, are gaining popularity and being effectively used to stimulate reproduction (Kutwal et al., 2017; Marimuthu, 2019). However, it has been suggested that synthetic hormones alone or in combination are more cost-efficient and effective.

**Carp Pituitary Extract (CPE).** Carp pituitary extract (CPE) is the most frequently utilised hormone for inducing spawning in fish worldwide and is used by culturists, especially the common carp, major Indian carp, and Chinese carp (Kahkesh et al., 2010). However, obtaining CPE is difficult, time-consuming, and inefficient, as it requires sacrificing many brood stocks to collect many pituitaries with a low success rate (Kutwal et al., 2017; Zohar & Mylonas, 2001). Furthermore, CPE is unpredictable in terms of quality, has a low activity level in the HPG axis, and could transmit diseases from donor to recipient fish. These problems have contributed to the increasing usage of human chorionic gonadotropin (hCG) to induce fish breeding (Dunham et al., 2000).

**Human Chorionic Gonadotropin (hCG).** Human chorionic gonadotropin (hCG) is a gonadotropin hormone discovered in the blood and urine of pregnant women in 1927 by Aschheim and Zondex (Elakkanai et al., 2015). This hormone is distinguished by wide market availability and high chemical purity that ensures its effectiveness (El-Hawarry et al., 2016). Human chorionic gonadotropin has been used to promote FOM and successfully induced spawning in numerous catfish species, including *Clarias gariepinus*, *Clarias batrachus*, and *Heteropneustes fossilis* (Kahkesh et al., 2010; Kather Haniffa & Sridhar, 2002; Inyang & Hettiarachchi, 1994; Sahoo et al., 2007).

**Luteinizing Hormone Releasing Hormone Analogue (LHRHa) or Salmon Gonadotropin-Releasing Hormone Analogue (sGnRHa).** Luteinising hormone-releasing hormone (LHRH) is a hypothalamic decapeptide, a synthetic analogue that activates the gonadotrophs of the pituitary to secrete gonadotropin (GTH) in teleost fish (Roy, 2016). The Linpe technique is an induced breeding technique that combines LHRHa or salmon gonadotropin-releasing hormone analogue (sGnRHa) with dopamine antagonists such as pimozide or domperidone (DOM). This technique is effective for silver carp, common carp, mud carp, black carp, grass carp, and loach (Peter et al., 1988). The Linpe method is efficient and reduces stress on brood stock as it only requires a single injection. Previous research revealed that a single injection of LHRHa or pellet implant successfully induces the spawning of marine species, such as milkfish, rabbitfish, sea bass, and mullet. However, this approach is ineffective for several freshwater species, including cyprinids (Roy, 2016). Other benefits of LHRHa include repeatability without loss of efficacy and the ability to produce LHRHa in pure form (Su et al., 2013). Thus, synthetic analogues of LHRHa are increasingly used to induce ovulation and spawning in teleost fish as the hormone is highly stable with precise dosage.

**Ovaprim.** Ovaprim is a synthetic hormone composed of a combination of the sGnRHa and DOM. Ovaprim is administered intramuscularly or intracoelomically and acts directly on the pituitary gland, boosting the production of gonadotropic hormones (GTH) while inhibiting the dopaminergic

inhibition of gonadotropin secretion. Ovaprim has been widely used in Asia and the United States to induce spawning in marine and freshwater fish (Abbas et al., 2019; Nuraini et al., 2017). Ovaprim has been reported to stimulate spawning in seurukan fish successfully (*Osteochilus vittatus*), selais (*Ompok hypophthalmus*), common carp (*Cyprinus carpio*), mali-mali (*Labiobarbus festivus*), lelan fish (*Osteochilus pleurotaenia*), yellowfin porgy (*Acanthopagrus latus*), stinging catfish (*Heteropneustes fossilis*), John's snapper (*Lutjanus johnii*), and mrigal carp (*Cirrhinus mrigala*) (Abbas et al., 2019; Mosha, 2018; Nuraini et al., 2017; Surnar et al., 2015; Watson et al., 2009). A study led by Watson et al. (2009) found that after injecting several species with Ovaprim, the average success rates increased to 50% (ovulation) and 54% (spermiation), and only 1.3% deaths were recorded, showing the efficiency of Ovaprim as hormonal therapy for artificial breeding.

## Comparative Study: Different Approaches of Artificial Hormones in Fish

**Study 1: Comparison of Luteinising Hormone Releasing Hormone Analogue (LHRHa) and Carp Pituitary Extract (CPE).** A study of the efficacy of using LHRHa to breed hybrid fry between channel female catfish (*Ictalurus punctatus*) and blue male catfish (*Ictalurus furcatus*) through LHRHa implant or injection and CPE injection (Su et al., 2013). In this experiment, LHRHa was diluted in 0.85% physiological saline and administered intraperitoneally at a starting dose of 20–30 g with 85% active ingredient per kg, followed by a resolving dose of 100–150 g/kg 12 h later. Meanwhile, LHRHa implants were administered into the muscle in a single dose of 75–100 g/kg body weight into the posterior and ventral to the dorsal, and the CPE was dissolved in 0.85% saline for 30–45 min before injection at a starting dose of 2 mg/kg or a dissolution dose of 8 mg/kg.

The result of this experiment was in line with the study by Hutson (2006) that evaluated the implant and injection of LHRHa in producing hybrid catfish from channel female catfish (*I. punctatus*) and blue male catfish (*I. furcatus*). According to the findings, LHRHa injection or implantation successfully induced breeding in channel female catfish and produced hybrid catfish embryos. High doses of injection and implants increased fecundity and the number of egg stripes in females. In this study, LHRHa implants produced significantly more ($P < 0.001$) fry/kg of broodstock than CPE. The implant method is more efficient as it requires only a single administration, which can reduce the stress during handling, compared to the LHRHa injection method, which requires two injections. Therefore, LHRHa is an excellent synthetic hormone for use in the ovulation of female catfish and the expansion of commercial hybrid catfish production.

Tan-Fermin and Emrata (1993) reported a significant variation in egg yield after female Asian catfish (*Clarias macrocephalus*) were induced with different

LHRHa administered with pimozide. A current study found that injection of 0.05-1.10 μg LHRHa in combination with 1 μg pimozide/g successfully promotes 100% ovulation in all *C. macrocephalus* females 16 h after treatment (El-Hawarry et al., 2016; Roy, 2016). Therefore, the appropriate inducing agent dose is critical for obtaining an optimal spawning egg number in catfish (Dhara & Das, 2018). A study by Kahkesh et al. (2010) showed that the use of LHRHa with CPE in the induced breeding of Benni (*Barbus sharpeyi*) achieves an excellent spawning success rate (87.5%), with high fertilisation (94.57%) and hatching (78.42%) rates.

**Study 2: Comparison of Ovaprim and Carp Pituitary Extract (CPE).** A study was performed with different doses of carp pituitary extract (CPE) and Ovaprim to observe the effects of inducing agents at varied temperatures and latency periods on the fertilisation and hatching rate of Asian catfish (*Clarias batrachus*) (Dhara & Saha, 2013). This experiment used gravid fish. For the CPE, the pituitary glands were obtained from newly deceased adult carp fish and used as a natural inducer. The procedures were carried out at three temperatures (26 °C, 28 °C, and 30 °C). Carp pituitary extract was administered at dosages of 40 and 120 mg/kg of body weight for females, while the males received 25 and 50 mg/kg of body weight. Meanwhile, the other group of females received Ovaprim at 0.8 and 2.0 ml/kg of body weight, and the males received 0.4 and 1.0 ml/kg of body weight through injection.

After 15 h of injection (latency period), the female fish were stripped to release eggs, the male testes were carefully removed, and eggs and sperms were collected. The results show that at 28 °C, the highest percentages of fertilisation (80%) and hatching (71%) were observed under high CPE treatment, and at latency periods of 14 and 15 h, perfectly smooth stripping and eggs without clusters were recorded. These results are similar to Srivastava et al. (2012), who used Ovaprim at 1.0–2.0 ml/kg body weight and a 14–18-hour latency period in *C. batrachus*. The inappropriate combination of dosage and latency period may lead to breeding failure in species, as shown in a study by Zonneveld et al. (1998). This study reveals that high dosages of Ovaprim and CPE result in significantly increased ($P < 0.05$) egg release, fertilisation, and hatching rates at all temperatures compared with those at low stimulant doses.

**Study 3: Sustain Release of Human Chorionic Gonadotropin (hCG) by Osmotic Pump.** In an *in vivo* study by Murugananthkumar et al. (2017) on *C. batrachus* and *C. gariepinus*, the osmotic pumps approach was used for the sustained release of the hCG inserted alongside the gonads in the peritoneal cavity and sutured with a 30-mm sterile catgut to prevent the backflow of the body's osmotic pump. The osmotic pump was loaded with 5,000 IU hCG or saline (as a control) and intraperitoneally implanted in catfish for 21 days during the pre-spawning phase (May–June). According to the manufacturer, the

osmotic pump used in this experiment can emit approximately 5 µl of solution each day when the fish are kept at constant ambient temperature. Previous studies proved that using an osmotic pump for the sustained release of hCG successfully promotes vitellogenesis and spermatogenesis in fish (Kagawa et al., 2009). Furthermore, the mean gonadosomatic index (GSI) and motility of the sperm for the group of males treated with hCG (*C. batrachus* and *C. gariepinus*) revealed a significant increase of 5% in the GSI ($P < 0.05$) compared with the control.

Meanwhile, the GSI in females receiving hCG implantation exhibited a significant increase of 41% ($P < 0.01$) compared with the control group. On the one hand, compared with the control group, the histology of hCG treatment in catfish testes demonstrated a normal development of spermatogenesis in germ cells towards the spawning phase in which the lumen was partly filled with spermatocytes and spermatids. On the other hand, the control group of female fish exhibited few development stages of oocytes. At the same time, the hCG treatment increased the quantity of fully developed immature oocytes and spawning-like growth in the fish ovary. This approach has been previously proven effective in sexually immature fish (Kagawa et al., 2009; Murugananthkumar, 2017).

Similar effectiveness was observed when using hCG to induce spawning in spotted murrels (*Channa punctatus*) and stinging catfish (*H. fossilis*). The effectiveness increased with the combination of Ovaprim hormone through the intermuscular injection method (El-Hawarry et al., 2016; Kather Haniffa & Sridhar, 2002). A natural hormone (i.e., hCG) and a synthetic hormone (i.e., Ovaprim) were injected into snakehead fish (*Channa marulius*) and successfully induced spawning in brood fish; the combination of Ovaprim and hCG effectively and successfully achieved up to 100% spawning rate (Hafeez-ur-Rehman et al., 2015). A significant enhancement in the fecundity of hCG implanted fish was reported through the experiment.

**Study 4: Comparison of Ovaprim and Combination of LHRH with hCG.** Dhas et al. (2017) assessed the effects of synthetic hormones on the reproductive performance of green chromide (*Etroplus suratensis*). This experiment used spawners that exhibited the second stage of gonadal development (contained spherical and opaque ovules) due to the onset deposition of the egg yolk. The ovary size ranged from 35 mm to 52 mm, the diameter was from 0.5 mm to 1.75 mm, and the ovaries were divided into three treatment groups: the hCG + LHRH, Ovaprim, and control (physiological saline) groups. The results showed that the hormone influenced the change in egg size in the oocytes, and the previtellogenic and mature phases depended on the type of hormone administered. The combination of hCG and LHRH produced the largest eggs, followed by Ovaprim and the control group (0.85% saline). The hCG + LHRH was the best treatment, with the highest fecundity and striping responses

(1.23 ml), highest fertilisation rate of 82.54%, and the highest hatching rate of 80.83%. Ovaprim followed with fecundity and stripping of 0.84 ml, fertilisation rate of 75.42%, and hatching rate of 73.69%.

A similar result trend was also observed in the males' treatment. The hCG + LHRH treatment enhanced the number of sperm cells (1,887 sperms/µl), followed by Ovaprim (860 sperms/µl) and the control (1,858 sperms/µl). The sperm motility improved by 89.78% when hCG + LHRH was administered, followed by Ovaprim (82.34%) and the control group (70.23%). This study revealed that the parameters were at the highest value under hCG + LHRH treatment, followed by Ovaprim treatment. Nwokoye et al. (2007) discovered that treating African giant catfish (*Heterobranchus bidorsalis*) with Ovaprim at 0.5 ml/kg body weight increased the fertilisation rate (98.31%). However, similar dosages were administered to induce spawning in spotted murrel (*C. punctatus*) and catfish (*Heteropneutes fossilis*), only producing low fertilisation rates of 75.0%, respectively (Kather Haniffa & Sridhar, 2002). The results indicate that the Ovaprim dosage should be varied depending on the species and the weight of the spawner (Dhas et al., 2017).

**Study 5: Ovarian Lavage with Sperm and Hormone Mixture (Sperm + Pituitary Gland Extract).** Ovarian lavage is an *in vitro* fertilisation that uses hormones to stimulate ovulation and spermiation. Ovarian lavage is a novel method in which hormone is administered intramuscularly or intraperitoneally while sperm is delivered into the ovarian lobes via a catheter (Müller et al., 2019). By using this method, female fish from group 1 were administrated with 5.0 mg/kg CPE, homogenised saline (0.7% NaCl) was injected intraperitoneally, and the eggs were fertilised using a traditional dry method. Fish from group 2 were injected similarly, but the sperms for the fertilisation process were injected into the ovarian lobe of the female. Females in group 3 were given homogenised 5 mg/kg of CPE with 2.0 ml/kg of sperm injected into each lobe of the ovarian cavity. The eggs in treatment group 1 were striped and fertilised with 2.0 ml/kg sperm. The eggs in treatment groups 2 and 3 were stripped, and fertilisation was activated by adding water.

The result showed that the ovulation ratio was 100% in group 1 with a latency time of 9.5 h, followed by group 2 with 85.7% and group 3 with 71.4% and a latency time of 10 h. This study's findings indicate that using sperm at a dose of 2.0 ml/kg in combination with 5 mg of a 2.0 ml dose of CPE resulted in successful ovulation and a high rate of fertilisation in silver catfish, *Rhamdia quelen* (Ittzés et al., 2020). The administration of hormones in combination with the injection of sperm from many males can be used in breeding programs to minimise bottleneck effects, synchronise egg production, avoid inbreeding, and reduce mortality. Modified gametes, such as polyploid or cryopreserved sperm, can also be used *in vitro* fertilisation (Müller et al., 2018). In captivity, a similar approach has been applied to spawning green-spotted puffer fish *(Dichotomyctere nigroviridis*). A

catheter was introduced into the oviduct at a 3 µl/g body weight rate as an alternative method of delivering hCG hormone to the fish (Watson et al., 2009).

**Study 6: Commercial Hormone in Combination with Hormonal Antagonist.** A study was conducted on African catfish (*Clarias gariepinus*) using homogenised carp pituitary in a physiological saline solution of 0.9% NaCl (4 mg of carp pituitary in one ml saline solution), hCG, synthetic LHRHa, and GnRHa with 10-mg benzyl alcohol. The hormone and hormone analogue were administered with 1 ml of dimethyl sulfoxide in combination with a DOM to measure the effects of using CPE, HCG, and LHRHa or GnRHa in combination with DOM on breeding performance. Seventy-two (72) females were randomly assigned to 10 treatment groups (i.e., T1, T2, T3, ... T10) in a complete randomised experimental design. Each experiment was performed in duplicate, with n = 4 fish in each experiment. Specific hormones were injected intramuscularly into the dorsal muscle of the female and male fish, and after 10 h of treatment, eggs and sperm were collected. A 2.5 ml of sperm was introduced to 100 g of eggs to fertilise the eggs for each treatment. Water was added to the active sperm, and the mixture was incubated at room temperature for 2 min before the eggs were rinsed with a 0.9% NaCl solution to complete the fertilisation process.

The fertilised eggs were washed with water and incubated in ventilated tanks at room temperature (28 °C) until hatching. The results revealed that the combination of hormones and a DOM effectively triggered ovulation in each *C. gariepinus*, with an ovulation rate of 100%. The highest ovulation rate was recorded from T4 (CPE + DOM: 100%), T6 (HCG + DOM: 100%), T8 (LHRHa + DOM: 100%), and T10 (GnRHa + DOM: 100%) followed by the ovulation rate in T3 (CPE: 87.5%) and T5 (HCG: 75%). The lowest ovulation rate was observed in T9 (GnRHa: 25%), followed by T7 (LHRHa: 12.5%). A study suggested that dopaminergic inhibition may contribute to the efficacy of hormone administration (Zohar et al., 1995). Mehdi and Seyed (2011) explained that due to the dopamine hormone suppressing pituitary secretion of GTH, the administration of DOM increases the effects of the GnRH analogue, resulting in a significant secretion of LH and ovulation. The combination of GnRHa and DOM can also successfully induce complete ovulation in female Malaysian mahseer (*Tor tambroides*) (Azuadi et al., 2011). Furthermore, a study showed that using DOM alone is ineffective in inducing complete ovulation in species, resulting in an insufficient response to stripping and showing that DOM without GnRHa is incapable of promoting maturation and ovulation (El-Hawarry et al., 2016).

## DISCUSSION

Various artificial hormones have been developed to address the reproductive system's failure and the dysfunction of gonadal development in captive fish. A previous study showed that using dopamine

antagonists (e.g., DOM) with other hormones could increase the fertilisation rate and produce good-quality seeds. In addition, domperidone does not cross the blood-brain barrier in goldfish and other teleosts fish by lowering the risk of negative side effects in treated fish (Peter et al., 1988). Another study states that in goldfish, common carp, and loach, sGnRHa is more efficient than LHRHa (Lin et al., 1988). However, other findings revealed that using LHRHa with pimozide or other dopamine antagonists was proven effective in promoting ovulation in female fish (Roy, 2016).

The use of hCG, CPE, GnRHa, and LHRHa with or without DOM effectively induces spawning (Table 1); past studies showed that the use of hormones with DOM could increase the success of fish farming (El-Hawarry et al., 2016). Apart from that, some studies suggested that combining GnRHa with DOM could boost GTH production, leading to increased sex steroids and the development of ovarian lobules (Sharaf, 2012). In addition, the use of GnRHa reportedly promotes maturation and spawning in various species, including African catfish (*C. gariepinus*), stinging catfish (*H. fossilis*), Siberian sturgeon (*Acipenser baeri*), brown trout (*Salmo trutta*), red seabream (*Pagrus major*), and starry flounder (*Platichthys stellatus*) (Alavi et al., 2012; Marimuthu, 2019; Okuzawa et al., 2016).

Aside from the dosage and type of hormone used, the delivery technique is also crucial in determining the success rate of artificial breeding. The implant method has potential because of the minimum handling of hormone insertion into fish, which could reduce handling stress and labour (Su et al., 2013). Furthermore, ovarian lavage is also preferred because it is a simple method for administering spawning hormone and eliminates injections for small species, such as spotted green pufferfish (*Tetraodon nigroviridis*) (Watson et al., 2009). Furthermore, the ovarian lavage approach is less time-dependent than the established *in vitro* fertilisation, and it could utilise sperm samples from chosen males depending on any factor to introduce a new generation (Ittzés et al., 2020).

The development of aquaculture technology, such as artificial breeding, has led to the application of technologies from low to high levels of development to increase the production of cultivated species. The use of breeding technology helps control reproduction in captivity, especially for wild and fish species that fail to reproduce and spawn naturally in captivity. Moreover, artificial breeding is essential for novel candidate species that are undomesticated and have a limited brood stock harvested from the wild to achieve sustainability in commercial aquaculture production. Some cultured fish may not spawn in captivity due to seasonal availability, lack of environmental cues, dysfunction, stress culture environments, or physio-physical factors, such as salinity and temperature. Therefore, well-established artificial breeding will help support the population of endangered species of commercial interest and those in the wild and captivity.

Table 1
*Comparative studies of different approaches to artificial hormones in fish*

| Comparative study | Fish species | Aim of study | Dosage | Results | References |
|---|---|---|---|---|---|
| Comparison of luteinizing hormone-releasing hormone analogue (LHRHa) and carp pituitary extract (CPE) | Channel female catfish, *Ictalurus punctatus* | LHRHa implant and injection and CPE injection | LHRHa injection: 20–30 g/kg and 100–150 g/kg bw<br>LHRHa implant: 75–100 g/kg bw<br>CPE injection: 2mg/kg bw | LHRHa implants produced significantly more ($P < 0.001$) fry/kg than CPE, and there are significant differences LHRHa injection and CPE injection ($P < 0.05$) | Su et al. (2013) |
| Comparison of Ovaprim and carp pituitary gland extract (CPE) | Asian female catfish, *Clarias batrachus* | Observe different doses of CPE and Ovaprim at varied temperatures and latency periods on the fertilization and hatching rate | CPE: 40 and 120 mg/kg of bw<br><br>Ovaprim: 0.8 and 2.0 ml/kg of bw | At 28°C, the greatest percentages of fertilisation (80%) and hatching (71%) were observed on high CPE treatment and Ovaprim at all temperature | Dhara and Saha (2013) |
| Sustain release of human chorionic gonadotropin (hCG) by osmotic pump | Adult catfishes, *Clarias batrachus* and *C. gariepinus* | *In vivo* induction of hCG by using an osmotic pump implanted intraperitoneally during the pre-spawning phase for 21 days LHRHa injection, 20–30 g/kg and 100–150 g/kg body weight | Osmotic pump: 5,000 IU hCG and saline (as a control) | GSI in females receiving hCG implantation revealed a significant increase of 41% ($P < 0.01$) compared to the control. Males also showed a positive outcome with a significant increase of 5% in the GSI ($P < 0.05$) when compared to the control | Muruganantkumar et al. (2017) |
| Comparison of Ovaprim and combination hormones of LHRH with hCG | Cichlid, *Etroplus suratensis* | To assess the effect of Ovaprim and combination hormone of hCG with LHRH on *E. suratensis* | hCG + LHRH: 1,000 IU +1 ml/kg fish<br><br>Ovaprim: 1 ml/kg fish | The use of the combination hormone hCG + LHRH resulted in the highest fecundity and striping responses (1.23 ml), highest fertilization rate of 82.54%, and the highest hatching rate of 80.83%, followed by Ovaprim with fecundity and stripping (0.84 ml), fertilization rate (75.42%), hatching rate (73.69%), and the lowest in control | Dhas et al. (2017) |

Table 1 *(continue)*

| Comparative study | Fish species | Aim of study | Dosage | Results | References |
|---|---|---|---|---|---|
| Ovarian lavage with sperm and hormone mixture (sperm + CPE) | Silver catfish, *Rhamdia quelen* | To study the effectiveness of the ovarian sperm injection method compared to the traditional method | CPE: 5 mg/kg bw<br><br>Sperm: 2.0 ml/kg bw | The ovulation ratio was 100% in group one, followed by group 2 with 85.7% and group 3 with 71.4%. The use of sperm at a dose of 2.0 ml/kg in combination with 5 mg of a 2.0 ml dose of CPE resulted in successful ovulation and a high rate of fertilization | Ittzés et al. (2020) |
| Commercial hormone in combination with a hormonal antagonist | African catfish, *Clarias gariepinus* | To study the effects of using CPE, hCG, LHRHa, and GnRHa with or without domperidone (DOM) | 0.9% NaCl: 4 mg carp pituitary in one ml saline solution<br><br>hCG: 4,000 IU/kg bw<br><br>LHRHa: 50 µg/kg bw<br><br>GnRHa: 40 µg/kg bw<br><br>DOM: 10 mg/ kg bw | A combination of hormones with a DOM effectively triggered ovulation in each *C. gariepinus*, with an ovulation rate of 100%. The highest ovulation rate was recorded from T4 (CPE + DOM: 100%), T6 (HCG + DOM: 100%), T8 (LHRHa + DOM: 100%), and T10 (GnRHa + DOM: 100%) followed by the ovulation rate in T3 (CPE: 87.5%) and T5 (HCG: 75%). While the lowest ovulation rate was observed in T9 (GnRHa: 25%), followed by T7 (LHRHa: 12.5%). The use of CPE, HCG, LHRHa or GnRHa together with dopamine antagonist (DOM) successfully induced ovulation in 100% of the experimental *C. gariepinus* broodfish | El-Hawarry et al. (2016) |

## FUTURE PROSPECT AND CONCLUSION

Artificial hormones could offer a promising technique to breed fish in captivity, ensuring seed availability, improving genetic loss, and reducing the dependency on wild-caught fingerlings. This review highlighted a comprehensive discussion and various types of artificial hormones, their delivery systems, and dosages used in artificial breeding within different fish species. In the future, more research should be focused on studying the latency period, thermodynamics, and metabolic pathways to ensure the developed hormones are safe, efficient, and effective for fish breeding.

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# Influence of Dietary Lysine Level on Growth Performance, Feed Efficiency, and Body Composition of Sangkuriang Catfish (*Clarias gariepinus* var. Sangkuriang) Fingerlings

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## ABSTRACT

The high consumer demand in Indonesia encourages catfish farmers to conduct an intensive culture. A low feed efficiency mainly occurs in cultivating Sangkuriang catfish resulting in poor growth. This condition might be caused by low lysine content, as lysine is an essential amino acid that the fish cannot synthesize. The present study aimed to investigate the effect of lysine supplementation in feed on protein digestibility, feed efficiency, and growth of Sangkuriang catfish (*Clarias gariepinus* var. Sangkuriang) fingerlings. The study used 270 Sangkuriang catfish with an average wet weight of 7.54 ± 0.13 g/fish. The experimental feed contained protein, energy, and amino acid, and then various doses of lysine were added to the experimental feed: (1) 0.0%, (2) 0.5%, (3) 1.0%, (4) 1.5%, (5) 2.0%, and (6) 2.5%. Weight gain (WG), protein digestibility (ADCp), the efficiency of feed utilization (EFU), relative growth rate (RGR), feed conversion ratio (FCR), protein efficiency ratio (PER), and protein retention (PR) of catfish were evaluated for 8 weeks. The results found that the supplementation of lysine in feed significantly ($P < 0.05$) influenced WG, ADCp, EFU, RGR, FCR, PER, and PR of Sangkuriang catfish fingerling. However, there was no significant effect ($P > 0.05$) on the SR of Sangkuriang catfish fingerling. The supplementation of 1% lysine/kg feed was the optimal dose to improve the feed efficiency and growth of Sangkuriang catfish fingerlings by 83.79% and 3.94%/day,

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respectively. Therefore, the supplementation of lysine could increase Sangkuriang catfish production.

*Keywords*: Catfish, efficiency, feed, growth, lysine

## INTRODUCTION

Sangkuriang catfish (*Clarias gariepinus* var. Sangkuriang) is a common cultivated freshwater fish in Indonesia with rapid growth, a high nutritional value, and the ability to adapt to the environment (Rachmawati et al., 2019). Feed is the most expensive component of intensive Sangkuriang catfish cultivation, accounting for up to 70% of total production costs per cycle (Rawles et al., 2011). The success of intensive Sangkuriang catfish cultivation depends on the feed quality. A high-quality feed contains an essential amino acid (EAA) profile related to the fish's needs. If the fish feed contains the appropriate amount of EAA, then the required protein for the fish will be fulfilled without a shortage or excess of amino acids (Miles & Chapman, 2008). Each fish species has a different requirement for protein and essential amino acids; thus, the protein content and number of essential amino acids in the feed will only provide effective results when they are within the range required by the fish (National Research Council [NRC], 2011). Amino acids could be used to maintain health and synthesize a new protein structure to improve fish feed efficiency and growth.

Conversely, lacking amino acids decreases feed efficiency and fish growth (L. Nguyen & Davis, 2016). The new protein structure is a protein that has an essential amino acid profile according to the needs of fish. Feeds with protein that has an essential amino acid profile according to fish requirements can increase protein digestibility, thereby increasing fish feed efficiency and growth. Conversely, if essential amino acids are deficient, protein digestibility, feed efficiency, and fish growth will be decreased (Ebeneezara et al., 2019).

The low effectiveness of feed consumption and the high feed cost are issues confronting Sangkuriang catfish cultivation. This problem is mainly caused by the lack of lysine content in the feed. The price of fish meals is increasing due to higher aquaculture production and the limited availability of fish meals (K. A. T. Nguyen et al., 2021). Plant-based protein sources are increasingly being used as an alternative source of protein in fish feed (Elesho et al., 2021). If the amount of lysine in the feed does not meet the needs of the fish, it causes feed inefficiency (Ebeneezara et al., 2019). The lysine requirement in fish differs depending on the fish species, and in the same species, the lysine requirement also varies depending on the growth stage of the fish (NRC, 2011). For example, el-Husseiny et al. (2017) reported that the lysine requirement of African catfish (*Clarias gariepinus*) was 1% of a dry diet. If the amount of lysine in the feed according to the needs of the fish can increase the digestibility of the ileum (intestines) so that nutrients can be absorbed quickly, the growth rate is high, and feed efficiency increases (NRC, 2011). As the main feed component,

the vegetable protein lacked amino acid lysine (Ebeneezara et al., 2019). Among all essential amino acids, lysine is a limited essential amino acid in most plant-based ingredients used for commercial fish feed (F. Zhou et al., 2010). NRC (2011) stated that lysine is an essential amino generally found in low amounts in the fish feed containing vegetable protein ingredients. One of the strategies to overcome the problems is the supplementation of lysine amino acid in the artificial feed of Sangkuriang catfish.

Lysine plays a role in protein deposition in the fish body, maintaining healthy blood vessels, producing antibodies, absorbing calcium, and repairing tissue damage (Robinson et al., 2007). In addition, lysine activates digestive enzymes, such as intestinal trypsin, to increase protein digestibility for growth (Zhang et al., 2021). Digested protein will then be stored in fish muscles through muscle hyperplasia or muscle size increase. Therefore, lysine is one of the factors that may regulate the hyperplastic process in fish skeletal muscle and thus become very important for optimizing fish muscle growth (Zhao et al., 2020). The addition of the lysine amino acid in the feed will quickly be used for metabolic processes compared to the addition of other amino acids (Farhat & Khan, 2013). Several studies have reported the requirement for the lysine amino acid for some species of fish, for example, in *Cirrhinus mrigala* (Ahmed & Khan, 2004), *Plectropomus leopardus* (Giri et al., 2009), *Pseudosciaena crocea* (Xie et al., 2012), *Heteropneustes fossilis* (Farhat & Khan, 2013), and *Trachinotus blochii* (Ebeneezara et al., 2019). However, until now, information on the requirement of lysine amino acids in Sangkuriang catfish is limited. The optimum lysine amino acid content in feed needs to be determined to develop an efficient fish feed for the growth of Sangkuriang catfish. Therefore, the present study aimed to investigate the effect of lysine supplementation in feed on protein digestibility, the efficiency of feed, and the growth performances of Sangkuriang catfish to improve the quality and increase Sangkuriang catfish production.

## MATERIALS AND METHODS

### Fish Preparation and Experimental Condition

Sangkuriang catfish fingerlings with an average weight of 7.54 ± 0.13 g/individual were obtained from a local commercial hatchery, Tambaksari Village, Rowosari District, Kendal District, Central Java, Indonesia. Then, the fish were acclimatized for 1 week before the experiment. During the acclimatization process, the fish were fed with feed that did not contain lysine by using the *ad satiation* method (until fish are satiety) three times a day, approximately at 8 a.m., 1 p.m., and 6 p.m. Before the experiment, fish were fasted to remove the remaining metabolism waste. Sangkuriang catfish used in the study were physically healthy, seen from the active swim, and had complete and normal body organs (Rachmawati et al., 2017). This research was conducted for 56 days.

**Research Design and Treatment**

A total of 270 Sangkuriang catfish fingerlings were divided randomly into six groups with three replications. Sangkuriang catfish fingerlings are maintained with a stocking density of 1 fish/L in a fiber tank (water capacity of 50 L) containing 15 liters of water, which is equipped with an aeration system where the temperature ranges between 26 °C - 29 °C, pH 7.0 - 8.5 and dissolved oxygen above 4 mg/L. A siphon was done to dispose of leftover fish feed and collect fish feces for digestibility of protein analysis to maintain the quality of the water. Siphoning was conducted before feeding the test at 8 a.m. and after feeding at 6 p.m. The fish per fiber tank biomass was weighed and calculated weekly for 8 weeks to determine weight gain and survival rate. The experiment was designed using a complete randomized design (CRD) with 6 different lysine content in a feed with three replications. The weight gain (WG), apparent digestibility of crude protein (ADCp), relative growth rate (RGR), efficiency of feed utilization (EFU), food conversion ratio (FCR), protein efficiency ratio (PER), retention of protein (PR), and survival rate (SR) were calculated based on following formulas:

$$WG\ (g) = Final\ body\ weight\ (g) - Initial\ body\ weight\ (g) \quad (1)$$

$$ADCp\ (\%) = 100 - \left\{\frac{100 \times Cr_2O_3\ in\ the\ fish\ feed}{\%\ Cr_2O_3\ in\ the\ feces} \times \frac{\%\ protein\ in\ the\ feces}{\%\ protein\ in\ diet}\right\}$$
$$\left\{\frac{100 \times Cr_2O_3\ in\ the\ fish\ feed}{\%\ Cr_2O_3\ in\ the\ feces} \times \frac{\%\ protein\ in\ the\ feces}{\%\ protein\ in\ the\ diet}\right\} \quad (2)$$

$$EFU\ (\%) = \frac{Final\ weight - Initial\ weight}{Weight\ of\ diet\ consumed} \times 100 \quad (3)$$

$$RGR\ (\%) = 100 \times \frac{(Final\ weight - Initial\ weight)}{(Times\ of\ experiment \times Initial\ weight)} \quad (4)$$

$$FCR = \frac{Feed\ intake\ (g)}{Body\ weight\ gain\ (g)} \quad (5)$$

$$PER = 100 \times \frac{(Final\ weight - Initial\ weight)}{The\ amount\ of\ diet\ consumed \times Protein\ content\ of\ diet} \quad (6)$$

$$PR = 100 \times \left(\frac{The\ total\ protein\ in\ fish\ body\ gain\ (g)}{The\ total\ protein\ consumed\ (g)}\right) \quad (7)$$

$$SR\ (\%) = 100 \times \left(\frac{Final\ count}{Initial\ count}\right) \quad (8)$$

**Diet Preparation**

The experimental diets with the same protein, energy, and amino acid content and different amounts of lysine amino acid were used. There were six lysine levels supplemented in the experimental diet: 0%, 0.5%, 1%, 1.5%, 2%, and 2.5% dry diet. Meanwhile, based on the percentage of lysine in the protein content of the feed experiment, the dose of lysine in the feed as treatment was equivalent to 3.72%, 5.13%, 6.52%, 7.94%, 9.39%, and 10.78% of feed protein (Table 1). The lysine used in this study was in the form of a brown powder with the brand name L-lysine hydrochloric acid (HCl) produced by Limited Liability Company Cheil Jedang (Indonesia). This experiment's range of the lysine level covers the lowest until the highest lysine content in Sangkuriang catfish fingerlings protein. Lysine is an essential amino acid for fish growth and physiological function (Deng et al., 2010). Among the ten essential amino acids, lysine is the first limiting amino acid in ingredients used in fish feed (Forster & Ogata, 1998). Feed was formed into pellets with a diameter of 4.2 mm, dried using an oven at 70 ºC for 3 h, and then stored in a refrigerator at 4 °C before being used. The experiment diet contained 34.1% – 34.8% protein and 4.1 - 4.3 kcal/g of energy. The formulation of the experimental diet (g/100 g feed) is shown in Table 1. The amino acid composition of the feed experiment and the amino acid composition of the Sangkuriang catfish fingerling are provided in Table 2.

Table 1
*Formulation of the feed experiment (g/100 g feed)*

| Ingredients | Feed experiment | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Fish meal | 24.50 | 24.50 | 24.50 | 24.50 | 24.50 | 24.50 |
| Casein | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 |
| Dextrin | 25.27 | 25.27 | 25.27 | 25.27 | 25.27 | 25.27 |
| Squid oil | 7.50 | 7.50 | 7.50 | 7.50 | 7.50 | 7.50 |
| Vitamin mix[1] | 1.31 | 1.31 | 1.31 | 1.31 | 1.31 | 1.31 |
| Mineral mix[2] | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| Lecithin | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 |
| Astaxanthin | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| Carboxymethyl cellulose (CMC) | 2.50 | 2.50 | 2.50 | 2.50 | 2.50 | 2.50 |
| Glutamic acid* | 2.50 | 2.00 | 1.50 | 1.00 | 0.50 | 0.00 |
| L-lysine | 0.00 | 0.50 | 1.00 | 1.50 | 2.00 | 2.50 |
| Amino acid mix[3] | 21.32 | 21.32 | 21.32 | 21.32 | 21.32 | 21.32 |
| Chromium oxide ($Cr_2O_3$) | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Total | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| Total lysine: % of diet** | 1.25 | 1.75 | 2.25 | 2.75 | 3.25 | 3.75 |
| % of protein*** | 3.59 | 5.06 | 6.52 | 8.06 | 9.39 | 10.84 |

Table 1 *(continue)*

| Ingredients | Feed experiment | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Proteins (%) | 34.8 ± 0.12 | 34.6 ± 0.15 | 34.5 ± 0.13 | 34.1 ± 0.10 | 34.6 ± 0.11 | 34.6 ± 0.13 |
| Lipid (%) | 9.4 ± 0.23 | 9.8 ± 0.22 | 10.5 ± 0.26 | 10.7 ± 0.20 | 10.7 ± 0.24 | 10.5 ± 0.21 |
| Ash (%) | 7.8 ± 0.30 | 7.8 ± 0.35 | 7.9 ± 0.32 | 7.8 ± 0.31 | 7.7 ± 0.33 | 7.8 ± 0.31 |
| Crude fiber (%) | 4.7 ± 0.09 | 2.5 ± 0.10 | 4.1 ± 0.13 | 2.5 ± 0.12 | 3.9 ± 0.11 | 2.0 ± 0.09 |
| Nitrogen-free extractives (NFE) | 33.0 ± 0.10 | 34.7 ± 0.14 | 32.3 ± 0.12 | 33.8 ± 0.13 | 32.7 ± 0.12 | 34.4 ± 0.12 |
| Energy (kcal/g fish meal)[4] | 4.1 ± 0.11 | 4.3 ± 0.12 | 4.2 ± 0.10 | 4.3 ± 0.11 | 4.3 ± 0.14 | 4.3 ± 0.11 |

[1]Vitamin mix (mg/100 g diet): choline chloride 900.0 mg/100 g diet; riboflavin 5.0 mg/100 g diet; biotin 0.6 mg/100 g diet; cyanocobalamin 0.01 mg/100 g diet; folic acid 1.5 mg/100 g diet; inositol 200 mg/100 g diet; niacin 2.0 mg/100 g diet; p-aminobenzoic acid 5.0 mg/100 g diet; menadion 4.0 mg/100 g diet; pyridoxin-HCl 4.0 mg/100 g diet; Ca-panthothenate 10.0 mg/100 g diet; calciferol 1.9 mg/100 g diet; α-tocopherol 2.0 mg/100 g diet; vitamin C- sty 120.0 mg/100 g diet; thiamin-HCl 5.0 mg/100 g diet; β-carotene 15.0 mg/100 g diet;

[2]Mineral mix (mg/100 g diet): potassium iodide (PI) 0.15 mg/100 g diet; iron(II) chloride tetrahydrate ($FeCl_3$, $4H_2O$) 166 mg/100 g diet; calcium carbonate ($CaCO_3$) 282 mg/100 g diet; zinc sulfate ($ZnSO_4$) 9.99 mg/100 g diet; manganese sulfate ($MnSO_4$) 6.3 mg/100 g diet; copper sulfate ($CuSO_4$) 2 mg/100 g diet; cobalt sulfate heptahydrate ($COSO_4 .7H_2O$) 0.05 mg/100 g diet; calcium dihydrogen phosphate [$Ca(H_2PO_4)$] 618 mg/100 g diet; magnesium sulfate ($MgSO_4$) 240 mg/100 g diet; potassium dihydrogen phosphate ($KH_2PO_4$) 412 mg/100 g diet;

[3]Refer to Table 2

[4] Total energy based on: protein = 4 kcal/g, lipid = 9 kcal/g, and carbohydrates = 4 kcal/g (NRC, 2011)

*The difference in the percentage of lysine was the treatment used in this study. While the difference in the presentation of glutamic acid is used in the feed formulation to obtain the protein content of the test feed, which is relatively the same

**% of diet: treatment in feed experiment

***% of protein: the total of lysine compared to the protein content of the feed experiment

Table 2
*The composition of amino acid in the feed experiment (g/100 g feed)*

| Amino acids | Feed experiment | | | | | | Sangkuriang catfish |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| **Non-Essential amino acids** | | | | | | | |
| Alanine | 8 .4 | 14.3 | 10.2 | 16.1 | 17.8 | 6.6 | 10.0 |
| Aspartic | 11 .2 | 16 .6 | 8.1 | 18 .6 | 21.4 | 6.3 | 8.0 |
| Glutamate | 11 .7 | 16.5 | 11.3 | 14.2 | 15.2 | 7.2 | 11.0 |
| Glycine | 14 .8 | 15.3 | 8.5 | 17.4 | 17.2 | 16 .9 | 8.3 |
| Proline | 18.8 | 16.3 | 14.6 | 17.9 | 18.7 | 17.5 | 14.1 |
| serine | 19.3 | 19.1 | 15.2 | 18.1 | 14.5 | 13.5 | 15.0 |
| Tyrosine | 12.9 | 14.7 | 11.4 | 13.2 | 13.8 | 8.7 | 11.0 |
| **Essential amino acids** | | | | | | | |
| Arginine | 16.9 | 15.4 | 10.8 | 15.6 | 12.4 | 8.4 | 10.3 |
| histidine | 7.6 | 6.4 | 4.0 | 6 .6 | 6.8 | 5 .3 | 3.7 |
| Isoleucine | 10.9 | 9.3 | 6.9 | 8.9 | 9.7 | 9.1 | 6.2 |

Table 2 *(continue)*

| Amino acids | Feed experiment | | | | | | Sangkuriang catfish |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| Leucine | 16.7 | 13.4 | 9.7 | 11.8 | 10.9 | 11.2 | 8.4 |
| Lysine | 16.3 | 14.7 | 12.9 | 14.6 | 15.5 | 16.7 | 12.3 |
| Methionine | 18.2 | 17.9 | 16.8 | 18.2 | 19.9 | 19.7 | 15.0 |
| Phenylalanine | 8.4 | 7.8 | 4.9 | 7.3 | 6.7 | 3.2 | 4.6 |
| Threonine | 18.9 | 15.2 | 13.5 | 17.4 | 17.3 | 16.6 | 12.0 |
| Valine | 10.8 | 9.6 | 8.0 | 8.9 | 10.4 | 10.5 | 7.1 |
| Tryptophan | 6.9 | 6.6 | 5.8 | 4.6 | 4.7 | 3.9 | 5.3 |

### Chemical Analysis

The composition of amino acids in the experimental diet was analyzed using a High-Speed Amino Acid Analyzer LA8080 AminoSAAYA (Hitachi High Technologies, Japan). A total of ± 1 mg of the sample was weighed, put in a closed tube, and hydrolyzed with 6 N HCl for 22 h at 110 ºC. After being filtered through 0.2 mm, the sample was ready to be injected into a High-Speed Amino Acid Analyzer LA8080 AminoSAAYA (Hitachi High Technologies, Japan) with an ion exchange resin column measuring 4.6 x 150 mm, temperature 53 ºC. The separation of amino acids was done using a gradient system with sodium citrate buffer solution (Merck KGaA, Germany) at pH 3.3, pH 4.3, and pH 4.9 with a flow rate of 0.225 mL/min. A reagent post column with a ninhydrin solution (Merck KGaA, Germany) at the speed of 0.3 mL/min was used to identify each acidic amino in length wave 570 nm and 440 nm (Ju et al., 2008). The Kjeldahl and Soxhlet methods determined the protein and fat content in the experimental diet and fish (El-Husseiny et al., 2017). The ash and water content of fish feed and body was defined based on Steffens's method (1989). The protein digestibility was analyzed using a spectrophotometer (Millipore, Merck KGaA, Germany) at 350 nm (Steffens, 1989).

### Statistical Analysis

Data on growth, feed efficiency, and survival rate of fish were analyzed using the analysis of variance (ANOVA) test and Duncan's multiple range test (DMRT) (Steel et al., 1997). Lysine requirement was determined by the regression method (Ziethoun et al., 1976). All statistical analyses were performed using the SAS 9.4 (SAS Institute Inc.) software for Windows (SAS, 2004).

## RESULTS

The initial average weight of fish was 7.54 ± 0.13 g/individual in all treatment groups. Table 3 shows that the lysine content in the feed significantly influenced ($P < 0.05$) WG, FI, ADCP, EFU, RGR, FCR, PER, and PR of Sangkuriang catfish fingerlings. However, the effect of lysine content in the fish feed was not significantly influenced ($P > 0.05$) SR of Sangkuriang catfish, indicated by an SR value of 100% during the study.

Table 3
*Data of WG, FI, $ADC_P$, EFU, RGR, FCR, PER, PR, and SR of Sangkuriang catfish fingerlings feed with different lysine content*

| Parameters | Feed Experiment | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Initial body weight (g) | $7.67 \pm 0.25^{a}$ | $7.54 \pm 0.28^{a}$ | $7.54 \pm 0.24^{a}$ | $7.54 \pm 0.25^{a}$ | $7.54 \pm 0.26^{a}$ | $7.41 \pm 0.25^{a}$ |
| Final body weight (g) | $19.64 \pm 0.12^{ed}$ | $21.42 \pm 0.10^{d}$ | $24.18 \pm 0.16^{a}$ | $21.86 \pm 0.13^{b}$ | $20.49 \pm 0.17^{c}$ | $18.58 \pm 0.12^{fed}$ |
| WG (g/fish) | $11.97 \pm 0.34^{e}$ | $13.88 \pm 0.32^{c}$ | $16.64 \pm 0.38^{a}$ | $14.32 \pm 0.39^{b}$ | $12.95 \pm 0.37^{d}$ | $11.17 \pm 0.35^{fe}$ |
| FI (g) | $23.89 \pm 0.10^{b}$ | $21.75 \pm 0.17^{c}$ | $19.86 \pm 0.19^{f}$ | $20.29 \pm 0.14^{e}$ | $21.63 \pm 0.18^{dc}$ | $24.32 \pm 0.16^{a}$ |
| ADCp (%) | $60.26 \pm 0.43^{e}$ | $75.64 \pm 0.46^{b}$ | $83.47 \pm 0.44^{a}$ | $70.22 \pm 0.42^{c}$ | $65.49 \pm 0.49^{d}$ | $55.34 \pm 0.41^{f}$ |
| EFU (%) | $50.10 \pm 0.37^{e}$ | $70.50 \pm 0.30^{b}$ | $83.79 \pm 0.35^{a}$ | $64.17 \pm 0.31^{c}$ | $59.54 \pm 0.35^{d}$ | $45.93 \pm 0.39^{f}$ |
| RGR (%/day) | $2.79 \pm 0.29^{e}$ | $3.39 \pm 0.25^{b}$ | $3.94 \pm 0.23^{a}$ | $3.29 \pm 0.27^{cb}$ | $3.07 \pm 0.22^{d}$ | $2.69 \pm 0.23^{fe}$ |
| FCR | $1.97 \pm 0.06^{e}$ | $1.42 \pm 0.03^{c}$ | $1.19 \pm 0.07^{a}$ | $1.32 \pm 0.04^{b}$ | $1.56 \pm 0.09^{d}$ | $2.18 \pm 0.03^{f}$ |
| PER | $1.44 \pm 0.52^{e}$ | $1.72 \pm 0.57^{d}$ | $2.43 \pm 0.51^{a}$ | $2.08 \pm 0.56^{b}$ | $1.85 \pm 0.51^{c}$ | $1.33 \pm 0.54^{f}$ |
| PR | $47.87 \pm 0.63^{d}$ | $52.11 \pm 0.68^{cb}$ | $57.56 \pm 0.62^{a}$ | $52.67 \pm 0.67^{b}$ | $45.84 \pm 0.61^{e}$ | $41.88 \pm 0.65^{f}$ |
| SR (%) | $100 \pm 0.0^{a}$ | $100 \pm 0.0^{a}$ | $100 \pm 0.0^{a}$ | $100 \pm 0.0^{a}$ | $100 \pm 0.0^{a}$ | $100 \pm 0.0^{a}$ |

*Note*. WG = Weight gain; FI = Feed intake; ADCp = Apparent digestibility of crude protein; EFU = Efficiency of feed utilization; RGR = Relative growth rate; FCR = Food conversion ratio; PER = Protein efficiency ratio; PR = Retention of protein; SR = Survival rate. Mean ± Standard deviation values with different *superscripts* showed significant difference ($P < 0.05$)

The WG, FI, EFU, RGR, FCR, PER, and PR of Sangkuriang catfish fingerlings which were fed with 2.25% of lysine (feed experiment 3), was significantly higher ($P < 0.05$) than in other treatments. On the other hand, fish-fed with 3.75% of lysine (feed experiment 6) had the lowest values ($P < 0.05$) on ADCP, EFU, FCR, and PR.

Proximate analysis of the fish body after receiving feed containing different lysine levels is shown in Table 4. There were significant differences ($P < 0.05$) in the crude protein of the fish body. However, between treatment groups, the fish body's dry matter, crude lipid, and ash levels were not significantly different ($P > 0.05$). Supplementation of 2.75%, 3.25%, and 3.75% of lysine in Sangkuriang catfish fingerlings feed significantly decreased crude protein content.

A regression polynomial analysis was conducted to determine the optimum amount of lysine in the Sangkuriang catfish fingerling feed. Based on the efficiency of feed utilization percentage, the optimum lysine in the feed was 2.37% of the dry diet (Figure 1) or 6.89% of feed protein. Sangkuriang catfish fingerlings fed with

Table 4
*Body chemical composition (g/kg) of Sangkuriang catfish*

| Composition | Feed experiment | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Dry matter | 28.36[a] | 28.61[a] | 29.13[a] | 28.55[a] | 28.78[a] | 29.43[a] |
| Crude protein | 16.66[d] | 18.03[b] | 19.86[a] | 17.96[c] | 15.86[ed] | 14.49[fde] |
| Crude lipids | 12.32[a] | 13.08[a] | 12.87[a] | 12.98[a] | 13.42[a] | 12.35[a] |
| Ash | 19.10[a] | 18.86[a] | 19.27[a] | 19.09[a] | 18.79[a] | 18.72[a] |

*Note*. Values within columns with the same letter are not significantly different ($P > 0.05$)

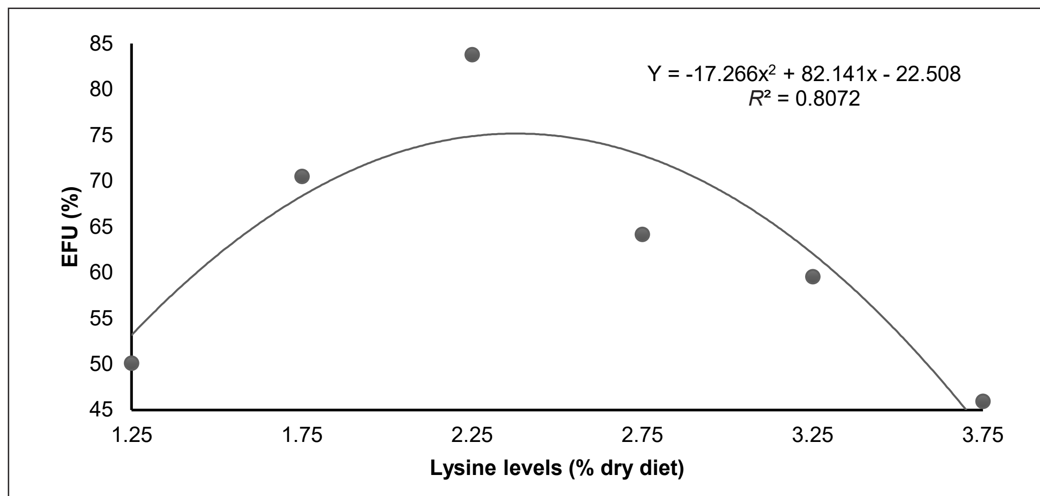


*Figure 1*. The correlation between lysine content in feed with the percentage of Sangkuriang catfish fingerling efficiency of feed utilization

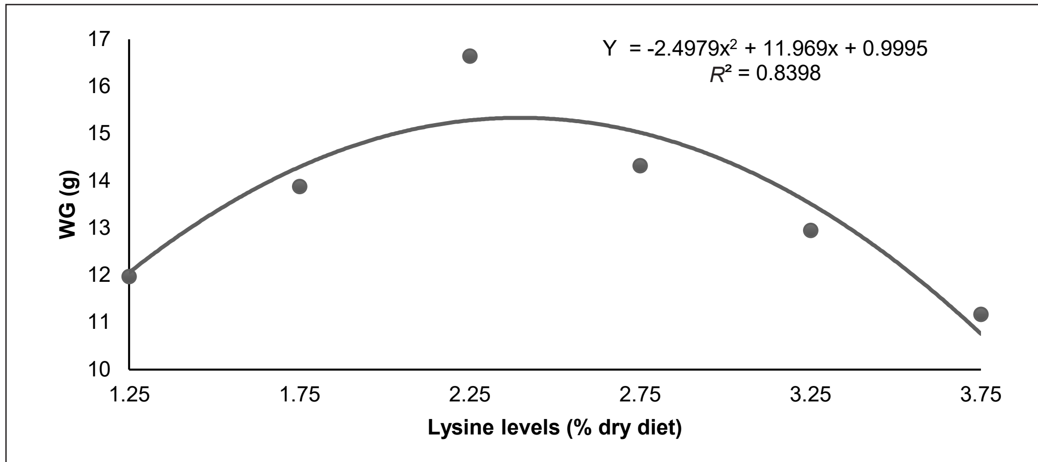


*Figure 2*. The correlation between the lysine content in feed with the percentage of Sangkuriang catfish fingerling weight increase

lysine addition of 2.75%, 3.25%, and 3.75% dry feed showed a decrease in crude protein content. Based on the weight of Sangkuriang catfish fingerling, the optimum lysine in the feed was 2.39% of the dry diet (Figure 2) or 6.93% of feed protein.

## DISCUSSION

The success in intensive fish cultivation highly depends on the quality of feed. A good quality fish feed contains protein according to the need of fish and contains a complete essential amino acid (EAA) profile (El-Husseiny et al., 2017). On the other hand, incomplete EAA profiles in fish diets have been linked to protein synthesis inhibition, leading to reduced growth (Hansen et al., 2007). Therefore, one of the efforts to complete EAA is to formulate fish feed with a balanced essential amino acid that meets the requirements of the fish (Khan & Abidi, 2011).

The response of fish growth to the number of amino acids in fish feed is the most widely used and accurate method for measuring the need for essential amino acids in fish (Bureau & Encarnacao, 2006). The need for amino acids can be statistically measured using polynomial regression, "broken-line" regression analysis, or the math-specific model depending on the fish growth response pattern (Dairiki et al., 2007). However, due to many factors, such as differences in the formulation of fish feed, size and age of the fish, genetics, feed management, and cultivation condition, the lysine need varies among fish species, even within the same species (Bureau & Encarnacao, 2006). According to Santiago and Lovell (1988), the percentage of feed protein determines the amino acid requirements of fish.

The requirement of lysine in the percentage of feed protein has been reported for some economically important fish, such as *Seriola quinqueradiata* (4.13%) (Ruchimat et al., 1997), *Chanos chanos* (4%) (Borlongan & Coloso, 1993), *Micropterus*

*salmoides* (4.9%) (Dairiki et al., 2007), *Cirrhinus mrigala* (5.75%) (Ahmed & Khan, 2004), and *Trachinotus blochii* (5.71–5.83%) (Ebeneezara et al., 2019). The levels of lysine required by the fingerling catfish Sangkuriang in our study are higher than those needed for the fish reported in the previous references. The value of necessary lysine in Sangkuriang catfish fingerlings obtained in this experiment was based on the percentage of feed protein which was relatively higher than the lysine requirement in the fish mentioned in the reference above. The best treatment was obtained by adding lysine to 2.25% of the dry diet, equivalent to 6.52% of protein. Variations in amino acid requirements can also be affected by the method of determination, environmental factors, and experimental design (Moon & Gatlin, 1991).

This study revealed that increasing lysine supplementation to 2.25% in the fish diet could enhance feed efficiency (EFU, FCR, PER, PR) and fish growth (WG and RGR). The Sangkuriang catfish fingerlings were shown to utilize the pure lysine added to the feed, indicating that the acid amino lysine is required for Sangkuriang catfish growth. The highest WG, ADCP, EFU, RGR, FCR, PER, and PR were found in Sangkuriang catfish fingerlings fed a diet containing 2.25% lysine. Fish will use a small amount of amino acid for energy and a larger amount for protein synthesis and metabolism, resulting in high fish feed efficiency and growth. Previous references were also similarly found on Japanese sea bass (Mai et al., 2006) and *Ictalurus punctatus* (L. Nguyen & Davis, 2016).

Our results showed that the addition of lysine above 2.25% stunted growth. Lower growth due to excess lysine in the diet has also been documented in several species (Bicudo et al., 2009; Mai et al., 2006; Q.-C. Zhou et al., 2007), but the mechanism is unclear. Furthermore, Ahmed and Khan (2004) suggested that decreased growth in fish fed high lysine was due to the antagonism effect of lysine to arginine, which disrupts the process of protein synthesis. Further research is needed to understand better the mechanism of interaction between lysine and other amino acids in fish. The addition of lysine in the feed above 2.25% showed a decrease in feed efficiency parameters (EFU, FCR, PER, PR). Lower fish growth due to excess lysine in feed has also been documented in several species (Bicudo et al., 2009; Mai et al., 2006; Q.-C. Zhou et al., 2007). The decrease in protein content fed with the addition of lysine above 2.25% can be caused by the excess amount of lysine used as an energy source. Suppose the amount of essential amino acids is excessive or there is a lack of non-protein energy sources (carbohydrates and protein). In that case, the body will use amino acids as an energy source (Lehninger & Nelson, 1993). Essential amino acids cannot be stored by the body and cannot be synthesized by fish (Ebeneezara et al., 2019). The effectiveness of pure amino acid use by fish was also reported to depend on the protein concentration of feed, with feed efficiency being low in high protein content and *vice versa* (Williams et al., 2001). It is also associated with the absorption of free amino acids much faster than protein amino

acids (Ahmed & Khan, 2004; Bicudo et al., 2009; Ebeneezara et al., 2019; F. Zhou et al., 2010; Xie et al., 2012).

The influence of the lysine content in feed on the fish composition proximate is provided in Table 4. The addition of lysine in the feed significantly affected ($P < 0.05$) the protein body of Sangkuriang catfish fingerlings but was not significant ($P > 0.05$) with the substance of the dried fish fat and ash. The highest crude protein content of Sangkuriang catfish showed in the feed containing a 2.25% lysine compared to other feed treatments. It is possible because the 2.25% lysine level in the feed has an EAA profile similar to the EAA profile of Sangkuriang catfish fingerling. Therefore, feed containing 2.25% lysine is the most suitable feed for Sangkuriang catfish fingerling. Akiyama et al. (1991) stated that the most appropriate feed had an amino acid profile similar to fish. In this way, fish will use very few amino acids for energy and more for body protein synthesis and metabolic functions resulting in high efficiency and growth of fish (Ebeneezara et al., 2019).

## CONCLUSION

The different lysine content in the fish feed significantly affects feed efficiency and growth of Sangkuriang catfish fingerling. Therefore, the supplementation of 1% lysine/kg feed was the optimal dose to improve the feed efficiency and growth of Sangkuriang catfish fingerlings by 83.79% and 3.94%/day, respectively. Therefore, the supplementation of lysine could increase Sangkuriang catfish production.

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# Evaluation of Carbon Stock, Nitrogen, and Phosphorus Contents in Forest Soil and Litter at Bintulu's *Acacia mangium* Chronosequence Age Stand Plantation, Sarawak, Malaysia

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## ABSTRACT

*Acacia mangium* is the major species used in the forest plantation industry due to its fast-growing feature. However, there is still a lack of research on the nutrient concentration, specifically nitrogen (N) and phosphorus (P), as well as carbon content in Malaysia's forest plantations. Hence, this study aimed to assess the total N and P concentrations in the soil and forest litter. Carbon content in different ages (Year 2, Year 4, and Year 9) of *A. mangium* plantation (together with a natural forest as a comparison) was also determined. This study was conducted in a Licensed Planted Forest, Bintulu, Sarawak, Malaysia. The natural forest was a control variable in this study. The Kjeldahl method was used to determine the total N. In contrast, dry ashing and double acid (Mehlich-1) methods were used to determine the total P in forest litter and available P in forest soil. The allometric biomass equations were used to estimate the carbon content. Total N in forest litter and forest soil was similar in all treatments. Total P in the Year 4 stand was significantly higher than in the Year 2 stand, yet, no differences were observed when compared with the control. Whereas soil available P showed no significant difference among all treatments. *Acacia mangium* stands recorded significantly lower total carbon content compared to the control. Old plantation stands contained much more total carbon stock than the younger stands. Also, deadwood is important in determining total carbon stock when it can account for almost 59% of above-ground biomass (AGB) carbon stock. This study revealed that forest

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plantations could function well in providing an adequate supply of available nutrients as well as have a potential role in carbon sink.

*Keywords*: *Acacia mangium*, biomass, carbon pool, soil N, soil P

## INTRODUCTION

Trees need nutrients to survive and grow healthily. Nitrogen (N) is one of the essential elements required by trees. In the plant metabolism process, N plays a prominent role. According to Leghari et al. (2016), protein, in which N is an important component, is involved in all critical processes in plants. Moreover, N also is a significant constituent of chlorophyll, enabling photosynthesis that can be found in many large parts of the plant cell. Nitrogen is vital for various physiological processes as it encourages tree growth and development as well as promotes the uptake and utilization of other nutrients such as potassium (K) and phosphorus (P). Phosphorus is considered one of the macronutrients. As every crop needs P for its growth, this nutrient has always been unavailable and unreachable in the soil. Consequently, this deficiency leads to the interruption in the photosynthesis process (H. Li et al., 2016), and plants' morphology, physiology, and metabolism may all be affected too (Hammond et al., 2004).

According to Intergovernmental Panel on Climate Change (IPCC) guidelines for national greenhouse gas inventories, there are five carbon pools of a terrestrial ecosystem, namely the above-ground biomass, below-ground biomass, the dead mass of litter, woody debris, and soil organic matter (Eggleston et al., 2006). Carbon sequestration in terrestrial ecosystems plays an important role in regulating gas exchange between plants, soils, and the atmosphere as it contributes to greenhouse gas emissions. Carbon exists in the atmosphere primarily in the form of carbon dioxide and approximately about 0.04%. The carbon stored in the plants is released back into the atmosphere during the respiration, decomposition of dead plant biomass and soil organic matter, or through combustion in nature (Vashum & Jayakumar, 2012).

Currently, forests cover 31% of the world's land, about 4.06 billion ha in total (Food and Agriculture Organization of the United Nations [FAO], 2020a). Of the total, 45% of them are in tropical regions. FAO (2020a) also found that due to land conversion, approximately 420 million ha of forests have been destroyed since 1990. Even so, different regions have different rates of forest depletion. Therefore, for tropical forests, statistics indicated that vegetation cover loss was estimated at 12 million hectares yearly between 2010 and 2018, up from 8.5 million hectares per year between 2002 and 2009 (Butler, 2019). As a result, forest plantations are established worldwide to cover the forest loss and act as substitutes for the natural forest. However, Liao et al. (2012) found that soil fertility in plantations is unlikely to return to that of natural forests, suggesting that replacing natural forests with plantations is a trend that should be avoided if environmental stability is maintained.

Koutika and Richardson (2019) revealed that *A. mangium*, a fast-growing tree native to parts of Indonesia, Papua New Guinea, and Australia, has been planted and imported into wet tropical lowland areas of Asia, South America, and Africa in recent times. The ability of *A. mangium* to deposit N-rich bases (low carbon to nitrogen [C/N] ratio) able to increase microbial activities, thus improving labile nutrients quantities in the soil (Bini et al., 2014), altering soil faunal, microbial, and bacterial populations, and promote crop or tree growth and forest productivity are key reasons for its extensive planting in commercial monoculture plantations or mixed plantings with other tree species or crops in areas with poor soil condition (Koutika & Richardson, 2019). In Malaysia, *A. mangium* has been planted widely since the 1960s to produce pulp and paper (Sukganah et al., 2005). Sabah was the first state in Malaysia to introduce this species in 1966 as a fire breaker species. Due to its rapidly growing and high survival rate, *A. mangium* was found to be suitable to be used in plantation establishments. Since then, the utilization of this species in forest plantations has expanded.

However, there is still a lack of research on the nutrient concentration, specifically N and P, as well as carbon content in Malaysia's forest plantation, particularly in Sarawak, Malaysia, although 43 Licensed Planted Forest (LPF) have been established. Currently, only a handful of a study conducted in Sarawak for soil properties and growth of *A. mangium* (Jusoh et al., 2017; K. L. Lee et al., 2015; Lim, 1986; Lim & Mohd. Basri, 1985; Nazeri et al., 2021, 2022; Tanaka et al., 2015). Only Tanaka et al. (2015) compared different age stands with adjacent existing natural vegetation stands. Nazeri et al. (2021) found that the diameter at breast height (dbh) and height of *A. mangium* stand increased over time. They found stand dbh increased from 15.5 cm at 3.7 years stand to 28.2 cm at 12.7 years stand. Meanwhile, tree height increased from 17.6 m to 27.6 m during the same period. Hence, the study aimed to assess the total N and P concentration in the soil and forest litter and carbon content in different ages of *A. mangium* plantation. The hypothesis is that the nutrient concentrations and carbon content assessed in the forest plantation are lower than the natural forests.

## MATERIALS AND METHODS

### Study Site

The study area was in an LPF in the Bintulu district, Sarawak, Malaysia. The total area of the LPF is 10,804 ha. This study area receives high annual rainfall with an average of 16.6 rainy days per month and 183 rainy days per year. Meanwhile, the type of soil found here is sandy loam, with a moderate amount of clay and silt (Chua, 2018). The sandstone mixed with shale and sand shale is the main component of bedrock found in the study areas, resulting in the Nyalau Formation (haplic Acrisols) formed during the Miocene. However, fertilisers were only supplied during the planting, and weeding was carried out twice only during the first year of establishment.

## Plot Establishment and Sampling

Sampling was conducted in four different stands, consisting of a natural forest area and three other age *A. mangium* stands (Year 2, Year 4, and Year 9). In each stand, four plots with a size of 20 × 20 m were established randomly. Figure 1 shows the conditions of each stand.

(a) Natural stand

(b) 2-year-old stand

(c) 4-year-old stand

(d) 9-year-old stand

*Figure 1*. Conditions of each study stand. (a) Natural; (b) 2-year-old; (c) 4-year-old; (d) 9-year-old

**Forest Litter.** Collecting forest litter was done using a 50 × 50 cm quadrat, placed randomly with the subplots of 10 × 10 m of each plot. Four samplings were carried out in each plot. Collected forest litter was put into a plastic bag according to their quadrat and brought to the laboratory for preparation and analysis.

Litter biomass and carbon estimation were conducted directly in the laboratory. First, the total dry weight of litter was converted into carbon by multiplying by 0.40 (Eggleston et al., 2006).

**Forest Soil.** Soil samples were collected from the same quadrat sampling as the forest litter samples. Coring with a height of 5 cm was used to collect soil samples. The first soil sample was taken from 0 to 15 cm soil depth, and the second sample was taken from 20 to 35 cm soil depth. This sampling was repeated in every quadrat in each plot. The soil samples were air dried before getting crushed manually and being sieved to remove large particles with a sieve tube of 2 mm in size.

Soil organic matter (SOM) was estimated by using a loss of weight on ignition (LOI) method (Schulte & Hopkins, 1996). The soil organic carbon (SOC) was calculated as 58% of SOM (Nelson & Sommers, 1996). The SOM, SOC, and soil carbon content (SCC) were calculated as:

$$\text{SOM (\%)} = \left(\frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}}\right) 100 \quad (1)$$

$$\text{SOC (\%)} = \text{SOM (\%)} \times 0.58 \quad (2)$$

$$\text{SCC (t/ha)} = \text{Bulk density} \times \text{SOC} \times \text{Soil volume} \quad (3)$$

**Tree Biomass.** Forest inventory was performed to obtain biomass estimation using the allometric model. Tree dbh in natural forest (control), four-year-old *A. mangium* stand (Year 4), and nine-year-old *A. mangium* stand (Year 9) was measured at 1.3 m above the ground of the tree in each plot with a diameter limit of 10 cm and above. While in a two-year-old *A. mangium* stand (Year 2), collar diameter was measured.

The allometric model by Kenzo et al. (2009) was used to calculate above-ground biomass (AGB) for natural forest stands. The allometric model used in this study for *A. mangium* stands of Year 4 and Year 9 has been developed by Adam and Jusoh (2018), while for *A. mangium* stands of Year 2 has been proposed by Thanh and Thu (2015). This present study uses an allometric model Niiyama et al. (2010) developed to estimate below-ground biomass (BGB) for the control site. Levan et al. (2020) developed the allometric model to estimate the BGB of *A. mangium* stands. The conversion factor of 0.47 was used to calculate carbon in AGB and BGB (FAO, 2020b).

**Deadwood.** All deadwood, standing or laying on the ground with a diameter limit of 10 cm above (FAO, 2020b), were identified and measured. Deadwood biomass included standing deadwood, fallen deadwood, and stumps. Measurement of dbh was used to estimate the biomass of standing deadwood. Meanwhile, fallen deadwood was divided into the tree and the main stem. The diameter size of both fallen deadwood

classes, as well as stumps, was determined to estimate the biomass.

Deadwood standing biomass was determined using the same AGB equations used for live stands. Whist fallen whole tree biomass was estimated using allometric models proposed by Kenzo et al. (2015) and Levan et al. (2020) for the control and different age of *A. mangium* stands, respectively. The stump biomass was estimated using equations suggested by Niiyama et al. (2010) for the control, while the estimation of stump biomass in *A. mangium* stands was using equations suggested by Levan et al. (2020). The total deadwood biomass was calculated by standing deadwood, fallen deadwood (whole tree and main stem), and stumps. Carbon content in deadwood is also calculated as 47% of the biomass followed FAO (2020b).

### Soil Chemical Analysis

The micro-Kjeldahl procedure was used to analyze N concentration in forest soil and forest litter samples (Horneck & Miller, 1998). At the same time, two different methods were used to determine P concentration: the double acid method (Mehlich, 1953) and the single dry ashing method (Lambert, 1976) for forest soil and forest litter analysis, respectively. The followings are the formula used to calculate the total P (mg kg$^{-1}$) and available P (mg kg$^{-1}$):

$$\text{Total P (mg/kg)} = \text{UV VIS reading} \times \left(\frac{\text{Mark up volume of sample after single dry ashing}}{\text{Weight of soil sample}}\right) \times \left(\frac{\text{Mark up volume of sample after developing blue colour}}{\text{The volume of sample used to develop blue colour}}\right) \quad (4)$$

$$\text{Available P (mg/kg)} = \text{UV VIS reading} \times \left(\frac{\text{Volume of double acid used}}{\text{Weight of soil sample}}\right) \times \left(\frac{\text{Mark up volume of sample after developing blue colour}}{\text{The volume of sample used to develop blue colour}}\right) \quad (5)$$

### Statistical Analysis

Data analysis has been done by using one-way analysis of variance (ANOVA) to see whether there is statistical confirmation that the related treatment means vary substantially. The significance level was determined using a probability standard of $p < 0.05$. When the ANOVA revealed statistically significant results, the means were compared using Tukey's studentized range test. Other than ANOVA, a *t*-test was performed to compare two groups' means, namely the upper and lower soil layers. Pearson's correlation coefficient test was also conducted to observe the relationship between the total N and P concentration with AGB, BGB, and litter biomass. Statistical Analysis System (SAS) (version 9.4) (SAS Institute, USA) was used for the statistical analysis.

## RESULTS

### Total Nitrogen

The control recorded the highest mean total N in its forest litter with a value of 0.39%, followed by Year 9, Year 4, and Year 2 (Table 1). However, no significant difference was observed among all four treatments (Table 1).

There was no significant difference among treatments for total soil N of both soil layers, as illustrated in Table 1. Year 2 recorded the highest values of total soil N in both soil layers. No significant difference was detected between the two layers (Table 1).

### Phosphorus

Year 4 recorded the highest mean total P in its forest litter with the value of 131.74 mg $kg^{-1}$, followed by Year 9, the control, and Year 2 (Table 1). A significant difference was detected between Year 4 and Year 2 (Table 1), whilst no difference was observed between the control and other *A. mangium* stands (Table 1).

In both soil layers, the control recorded the highest mean for available P concentration (Table 1). However, the difference between treatments is not significant (Table 1). The upper soil layer recorded more than 50% higher available P than the lower soil layer (Table 1).

### Carbon Stock

Results indicated a significant difference in AGB carbon stock in the control compared with three other treatments in *A. mangium* stands (Table 1). Besides, within the *A. mangium* stands, AGB carbon stock in Year 2 and Year 4 were statistically similar but significantly lower than in Year 9 (Table 1).

The control recorded significantly higher BGB carbon stock than *A. mangium* stands of Year 2, Year 4, and Year 9 (Table 1). Results also indicated a significant difference in BGB carbon content between *A. mangium* stands in Year 2 and Year 9 (Table 1).

There was a significant difference in deadwood carbon stock between the control and *A. mangium* stands of Year 2, Year 4, and Year 9 (Table 1). Within the *A. mangium* stands, deadwood carbon stock in Year 2 and Year 9 were statistically similar but significantly lower than in Year 4 (Table 1).

The mean comparison indicated that litter carbon in *A. mangium* Year 2 and Year 4 was similar but significantly lower than the control (Table 1). Meanwhile, litter carbon of Year 9 was significantly higher than another three stands (Table 1).

Results indicated a significant difference in soil carbon at both depths (Table 1). The control had significantly higher SCC than another three acacia stands at both soil depths (Table 1). However, at a depth of 0–15 cm, SCC in *A. mangium* Year 4 is significantly higher than in Year 2 and Year 9 (Table 1). While at a depth of 20–35 cm, the mean comparison indicated that the SCC in *A. mangium* Year 4 and Year 9 were similar but significantly higher from Year 2 (Table 1).

The total carbon stock in the control was statistically higher than the three ages

Table 1
*Site chemical properties of logged over and* Acacia mangium *stands at different ages*

| Properties | Control | Year 2 | Year 4 | Year 9 |
|---|---|---|---|---|
| Soil pH (water) (0–15 cm) | $4.44 \pm 0.29^{a2}$ | $4.36 \pm 0.27^{a2}$ | $4.21 \pm 0.09^{a2}$ | $4.66 \pm 0.15^{a2}$ |
| Soil pH (water) (20–35 cm) | $4.66 \pm 0.17^{a1}$ | $5.02 \pm 0.14^{a1}$ | $4.86 \pm 0.06^{a1}$ | $4.99 \pm 0.26^{a1}$ |
| Total N in forest litter (%) | $0.39 \pm 0.23^{a}$ | $0.25 \pm 0.05^{a}$ | $0.32 \pm 0.08^{a}$ | $0.35 \pm 0.13^{a}$ |
| Total N in soil (%) (0–15 cm) | $0.06 \pm 0.00^{a1}$ | $0.08 \pm 0.01^{a1}$ | $0.06 \pm 0.01^{a1}$ | $0.06 \pm 0.01^{a1}$ |
| Total N in soil (%) (20–35 cm) | $0.08 \pm 0.02^{a1}$ | $0.08 \pm 0.03^{a1}$ | $0.07 \pm 0.02^{a1}$ | $0.06 \pm 0.02^{a1}$ |
| Total P in forest litter (mg kg$^{-1}$) | $53.47 \pm 34.98^{ab}$ | $34.21 \pm 10.52^{b}$ | $131.74 \pm 63.14^{a}$ | $117.62 \pm 41.39^{ab}$ |
| Available P in soil (0–15 cm) (mg kg$^{-1}$) | $1.23 \pm 0.19^{a1}$ | $0.82 \pm 0.41^{a1}$ | $0.60 \pm 0.26^{a1}$ | $0.79 \pm 0.47^{a1}$ |
| Available P in soil (20–35 cm) (mg kg$^{-1}$) | $0.53 \pm 0.46^{a2}$ | $0.25 \pm 0.13^{a2}$ | $0.24 \pm 0.12^{a2}$ | $0.29 \pm 0.37^{a2}$ |
| Above-ground C (t ha$^{-1}$) | $104.71 \pm 15.46^{a}$ | $2.11 \pm 0.44^{c}$ | $7.67 \pm 1.48^{c}$ | $29.78 \pm 2.66^{b}$ |
| Below-ground C (t ha$^{-1}$) | $37.97 \pm 6.69^{a}$ | $0.52 \pm 0.11^{c}$ | $2.23 \pm 0.42^{bc}$ | $8.33 \pm 0.73^{b}$ |
| SCC (0–15 cm) (t ha$^{-1}$) | $61.09 \pm 1.80^{a1}$ | $39.49 \pm 3.71^{d1}$ | $55.25 \pm 2.56^{b1}$ | $50.76 \pm 2.80^{c1}$ |
| SCC (20–35 cm) (t ha$^{-1}$) | $50.99 \pm 1.31^{a2}$ | $35.78 \pm 3.91^{c2}$ | $45.51 \pm 1.48^{b2}$ | $45.03 \pm 1.11^{b2}$ |
| Litter C (t ha$^{-1}$) | $5.05 \pm 0.62^{b}$ | $3.35 \pm 0.90^{c}$ | $2.95 \pm 0.46^{c}$ | $11.74 \pm 2.99^{a}$ |
| Deadwood C (t ha$^{-1}$) | $273.31 \pm 49.51^{a}$ | $59.45 \pm 16.62^{c}$ | $185.05 \pm 27.66^{b}$ | $37.89 \pm 1.33^{c}$ |
| Total C (t ha$^{-1}$) | $533.12 \pm 103.50^{a}$ | $140.70 \pm 36.25^{d}$ | $298.67 \pm 81.59^{b}$ | $183.51 \pm 35.24^{c}$ |

*Note*. Means with same lowercase letters indicated no significant difference between Tukey's HSD test treatments at $p < 0.05$. Meanwhile, means with the same numbers indicated no significant difference between soil layers (0–15 cm and 20–35 cm soil depths) by independent *t*-test at $p < 0.05$

of *A. mangium* stands (Table 1). Meanwhile, within the *A. mangium* stands, the total carbon content in Year 4 was significantly higher than in Year 2 and Year 9 (Table 1).

### Relationships Between Phosphorus and Biomass

Available soil P at 0–15 cm depth was found to have a positive and significant correlation with AGB ($r$ = 0.5520, $p$ < 0.05) and BGB ($r$ = 0.5559, $p$ < 0.05). Meanwhile, a negative correlation was observed between total P in forest litter and available soil at 20–35 cm depth ($r$ = -0.5178, $p$ < 0.05).

## DISCUSSION

### Total Nitrogen in Forest Litter

*Acacia mangium* is a leguminous species overflowing with N due to its symbiotic N fixation capability (Arai et al., 2008). Therefore, it is expected that high N concentration can be found in the litterfall of acacia stands. However, our study revealed that the total N of forest litter in acacia stands is equivalent to the control (Table 1).

Tamm (1995) explained that natural forest ecosystems often have well-balanced nutrient cycles with negligible nutrient loss. Nevertheless, aspects such as forest successional stage, water regime, mineralogy and rate of soil weathering, external inputs of nutrients and hazardous chemicals, nitrogen fixation, denitrification, nitrification, and natural or anthropogenic disturbances all have an impact on the balanced cycle. The similar total N of natural forest (the control) with acacia stands in our study is contributed by this condition. The control has established a rooting network that has enabled the nutrient cycle, particularly the N cycle, to be conducted at a peak level. Thus, an adequate amount of N could be supplied to the mixed species that stands in control, resulting in a similar concentration of total N in forest litter in all treatments.

Our finding was consistent with previous studies (Maro et al., 1991; Ngaba et al., 2019). The highest total N concentration was observed in the natural forest (the control), with no remarkable difference between the treatments. It is owing to the mixed tree species found in the plot. Compared to monocultures, mixed rainforest roots access several N pools and reduce N absorption from a single source, for instance, ammonium ion ($NH_4^+$). It is due to the great plant diversity, which can lead to species complementarity in N resource usage based on individual preferences and capacities to absorb N (Wang & Macko, 2011). In addition, Britto and Kronzucker (2013) pointed out that plants use a variety of ways to get N from the soil. There are species that, for example, take up N as nitrate ($NO_3$), $NH_4^+$, or amino acids, obtain N sources at different soil depths, or are linked with N fixing organisms and mycorrhizal fungi for N supply (Brundrett & Tedersoo, 2018), resulting to an adequate supply of N to the mixed tree species.

Total N is directly proportional to the ages of the stands (Table 1) as the older the tree, the greater the surface area of its roots, increasing the nutrient acquisition by the tree (Näsholm et al., 2008), which elucidates the

concentration of total N in each treatment. Other than that, the amount of N supplied by the soil affects the concentration of total N in the plants (Morgan & Connolly, 2013). As all the plots studied showed a similar total N concentration in their forest litters, the amount of N supplied by the soil might be similar. According to Baldwin (1975), the total amount of diffusible nutrients, the pace at which the nutrient may move, and the distance it needs to travel to a root surface are the main parameters influencing nutrient delivery to a specific plant. The rooting volume is swiftly decreased, and the amount absorbed is completely determined by the quantity in the rooting volume. Thus, this study area is assumed to have the equivalent amount of nutrient delivery to the plants from the soil, describing the similar total N in all treatments (Table 1).

**Total Nitrogen in Forest Soil**

Total N in forest soil is expected to be similar between *A. mangium* plantation and natural forests, as found by Yamashita et al. (2008). It corresponds with our present study, which recorded the similar soil N in both layers in all treatments (Table 1).

Year 2 has the highest total N in forest soil of both soil layers (Table 1) due to the accumulation of wood residues during previous tree felling. Siregar et al. (1999) mentioned that all slash left remains, debris that has piled and some of which has decayed, and understorey that stays in place will all play an essential part in the site's nutritional budget. In addition to wood residues, the size of the roots also has resulted in high N concentration in Year 2. Smaller volume, size, and structure may result in lower nutrient adsorption from the soil; thus, higher concentrations of nutrients can be expected in younger stands. It is well-known that the bigger the tree size, the bigger the roots. Therefore, root size and structure significantly affect plant nutrient absorption efficiency (Fitter et al., 1991). Generally, Germon et al. (2018) disclosed that a high capacity of N absorption could be gained from the greater root length and root area of a tree. Simulations on this aspect have revealed that root structure influences the volume of soil from which nutrients may be extracted (Fitter, 1987; Fitter et al., 1991) as the higher the volume of soil accessed, the more nutrients can be absorbed by the roots. Thus, explaining stand age does influence the concentration of N in the soil. The youngest stand (Year 2) recorded higher total soil N may be related to lower N uptake, while older stands with more established root networks have higher N uptake, leaving less N concentration in the soil.

Even so, there was no significant difference observed between the treatments. It is due to the contribution of the forest litter itself. *Acacia mangium* is known for its capability to fix N. Thus, the concentration of N in the *A. mangium* tree would be high. The N will be released into the soil when the plant parts, such as stem twigs, primarily leave fall to the ground and undergo decomposition.

The total N concentration in forest soil recorded in the current study corroborated

with a study conducted in South Sumatra, Indonesia, by Yamashita et al. (2008) as well as a study held in Sabah, Malaysia, by Inagaki and Titin (2009).

Xue and An (2018) revealed in their study that a higher total N could be found in the surface soil layer compared to the deeper soil layer. However, our analysis indicated a different result, where both soil layers have a similar total N in the soil (Table 1). This situation may be related to the leaching process in the below-ground zone. Because of the frequent and severe rainstorms (Halmi & Simarani, 2021), higher temperatures, and high carbonic acid concentration in soil (Johnson et al., 1975), leaching losses are typically believed to be greater in the humid tropics than in temperate countries. Therefore, it is expected that soil leaching is rather high in our study site, causing the accumulation of N in the deeper soil layer as the concentration of total N is equivalent in both soil depths.

**Total Phosphorus in Forest Litter**

*Acacia mangium* in Year 4 showed the highest mean of litter P might be due to root morphology, such as root length and root diameter. Variations in root morphology would result in various amounts of P uptake by plants. Plants can increase their ability in soil exploration if their roots are long (Batista et al., 2016) and when greater surface areas for nutrient absorption are covered, leading to higher nutrient acquisition. In contrast, a shorter length of roots would limit the accessibility of the roots to the available nutrients in the soil. It explains why Year 4 has a higher total P concentration in litter than Year 2, as these stands consisted of juvenile *A. mangium* trees with shorter and smaller roots compared with older *A. mangium* in Year 4, which would have longer and bigger roots. Laclau et al. (2013) found higher root density in older *Eucalyptus grandis* plantations.

Furthermore, Y. Li et al. (2018) mentioned that large trees have an abundance of nutrient reserve, but young trees have a limited deposit. Hence, large trees absorb higher P concentrations than small trees. Besides, K. L. Lee et al. (2015) noted that the sturdy development of *A. mangium* would consume a massive amount of nutrients. The four-year-old *A. mangium* stand has these traits, which are large and vigorous development, resulting in a higher P requirement than Year 2.

Forest plantation is expected to have a lower total P accumulated in the litter than natural forest, as found by Yang (2005). However, Mani and Cao (2019) also reported that the natural forest they studied had a higher total P which ranged from 600 to 700 mg $kg^{-1}$. Similarly, the control recorded a higher total P in the forest litter in the current study.

However, our study recorded that the total P in both acacia stands and the control forest litter were similar (Table 1). It was probably owing to the physiological properties of the species planted itself. Fast-growing species like *A. mangium* can use nutrients more efficiently than slow-growing species (Cossalter & Pye-Smith, 2003;

Inagaki & Tange, 2014). A response made by a plant to alter its development and metabolic process as well as interact with soil microbes that could provide soluble P, called P starvation response (Isidra-Arellano et al., 2021), combined with the nutrient use efficiency, led to the equivalent total P between the acacia stands and the control.

Other than that, the symbiosis relationship between the acacia trees and soil microbes has enabled the similar uptake of P by the trees in the natural forest. Plants are known to evolve, especially when the nutrients are limited (Morgan & Connolly, 2013). Karandashov and Bucher (2005) described that this type of symbiosis promotes plant P absorption from the soil by expanding the absorptive surface area of the root. The associated mycorrhizal hyphae bridge the internal root environment and the region beyond the depletion zone, allowing the plant to obtain substantially more P via its symbiotic partner than it could alone (Chiu & Paszkowski, 2019). Hence, this explains why the total P uptake by the *A. mangium* trees is not lower but similar to the natural forest, as the soil microbes assist this species in improving its nutrient absorption rate.

**Available Phosphorus in Forest Soil**

Although there was no significant difference between treatments for both soil layers, the control recorded the highest soil available P in both layers, similar to previous studies by Chauhan et al. (2008). The higher P is probably due to the heterogeneity in the soil organic matter. As the control consists of mixed-species trees, the forest litter would also consist of multi-species organic matter that would contribute to higher P availability in the soil. Koutika et al. (2020) found that the mixed-species forest stands accumulated more P in organic forms, limiting P losses through leaching and helping to the medium-term sustainability of P requirements. Moreover, Cissé et al. (2021) revealed that mixed species of tree litters would facilitate a higher decomposition rate than pure litters. With the high proportion of tree biomass in the study site, there will be more forest litter that would supply nutrients needed by the plants. Therefore, this would increase nutrient cycling in the soil, which elucidates the higher P concentration in the control soil.

Meanwhile, Binkley (1992) mentioned that because root nodules' biological N fixation process mainly depends on P, legumes consume more P than non-leguminous plants. However, according to Mitran et al. (2018), to optimize the functionalities of legumes in the N fixation process via reciprocal symbiotic connection with certain bacteria in the soil, legumes require additional P, which is essential for energy transformation in nodules. Furthermore, P is important for root formation, nutrient absorption, and legume crop growth, which makes the P availability in the soil lower in the acacia plantation but not significantly different from the control.

Approximately 90% of flora species could establish a mutual connection with the arbuscular mycorrhizal fungi (AMF) (Smith & Read, 2008; Zhu et al., 2010). These fungi supply a variety of pivotal ecological

functions, including enhancing plant nutrition, stress responses and endurance, soil properties, and fertility (Chen et al., 2018). The plants and the AMF benefit each other as the AMF will be supplied with carbon by the host plants while, in return, the AMF facilitates their hosts in nutrient absorption, including P (Ferrol et al., 2004). Thus, this elucidates the similarity of available soil P in all treatments as *Acacia* species and multi-species in control rely on the AMF to increase their P availability in the soil.

The topsoil is generally more fertile than the lower layers (Table 1) and contains the most plant roots. Therefore, tree litter that falls onto the ground would supply nutrients to the soil, primarily into the upper layer. Litterfall quality would affect the soil nutrient availability (Ge et al., 2013). Soil organisms will decompose those plant residues under certain environmental conditions related to the temperature, moisture, and soil properties (Bot & Benites, 2005), which would contribute to the nutrients in the soil. Plus, the upper soil layer has the highest concentration of roots, with more than 90% of the roots being found in the surface soil due to nutrient absorption (Pierret & Moran, 2011). Consequently, higher P concentration can be observed in the upper layer (Table 1) as many roots can be spotted here during the P absorbance through diffusion.

This study proved that the tree biomass contributed significantly to the available P in the soil. The high amount of biomass will create more organic matter (Bot & Benites, 2005) that will eventually decay and be converted into available nutrient forms for plant uptake by related microorganisms.

Chatzistathis and Therios (2013) concluded that soil nutrient availability is one of the factors that could increase tree biomass. Therefore, the higher the soil's available P, the more biomass can be produced. Halomoan et al. (2015) reported the same findings where the tree biomass increased directly proportional to the amount of P available. Phosphorus is vital for the biological N fixation and the tree growth of leguminous plants. Hence, its deficiency would influence not only the N fixation process but also the development of a tree (Le Roux et al., 2008; Power, 2010). According to Lynch et al. (1991) as well as Radin and Eidenbock (1984), leaf expansion is very susceptible to P deficit, and it has been proposed that impairment in leaf expansion is a reaction to low P availability in P-deficient plants. Therefore, an adequate amount of P will result in an increase in leaf area, which will improve light interception and biomass output. The relationship between available P and the tree biomass works both ways as they benefit each other in the nutrient cycle.

In the current study, total P in the litter negatively correlated with the soil available P (especially at 20–35 cm depth), indicating that the high concentration of total P in the litter would lead to a lower concentration of available P in soil. It might be related to, among others, plant uptake, which in turn results in higher P concentration in the litter (Prasad & Chakraborty, 2019).

### Tree Biomass Carbon

The control had significantly higher tree biomass carbon (AGB and BGB) compared to all *A. mangium* stands (Table 1). The results also showed the increase of tree biomass carbon in *A. mangium* stands from youngest to oldest. This pattern can be explained by tree biomass and carbon content related to stem size.

According to Niiyama et al. (2010), the tree size has good linearity to estimate BGB carbon. This consequence was also reported by Kueh et al. (2012, 2013), who stated that the larger the diameter size of the tree, the more biomass and carbon storage capacity was held. Moreover, the result of this study explains that the tree biomass carbon of *A. mangium* increased significantly with increasing stand age. This finding is consistent with those reported in other previous studies (Herdiyanti & Sulistyawati, 2009; Levan et al., 2020; Palma, 2014).

The total tree biomass carbon of *A. mangium* stands in this study was 2.62 t/ha, 9.90 t/ha, and 38.11 t/ha in the 2-, 4- and 9-year-old stands, respectively. This value is much lower than values reported by Leva et al. (2020) for *A. mangium* plantation in South-eastern Vietnam, where the total tree biomass carbon was 34.53 t/ha, 62.21 t/ha, and 101.52 t/ha for the 4-, 7-, and 11-year-old plantations. It could be due to various factors influencing the difference in total tree biomass carbon, such as the environmental condition of the area and the various forest management practices used. Apart from that, this may be due to different biomass equations, which influence the estimated value of carbon content in a forest area. According to Temesgen et al. (2015), studying variations in allometric forms based on species, climatic, and edaphic factors is important to understand these variations in coordinated biomass equations because it is also sensitive to a larger number of measurable tree components.

### Deadwood Carbon

This study indicated that the control contained higher deadwood carbon than all *A. mangium* stands (Table 1). It could be due to the control being logged over the forest, which produces more deadwood due to the reduced effect of the live trees from previous logging activity (Pfeifer et al., 2015). In addition, logging removed large trees and caused residual damage to other live trees. According to Matangaran et al. (2019), stand damage after logging activities dramatically affects the composition of tree species in logged-over forest areas, and higher post-logging death rates have been documented, as illustrated by the accumulation of deadwood from stand damage after logging. Moreover, the control is a natural forest comprising various tree species compared to *A. mangium* stands with monocultural tree species. Hence, various tree species can contribute to the high amount of deadwood with different decomposition rates (Pfeifer et al., 2015).

Besides, within *A. mangium* stands, Year 4 recorded higher contribution of deadwood obviously in fallen deadwood compared to other two different stands. Another proposition is site condition factors,

such as the higher terrain steepness and old skid trails from previous logging activities. Higher terrain steepness causes unstable soil conditions for standing living trees, increasing the risk of landslides. It is the same with trees planted in the old logging trails area. It is due to the soil compaction affecting the development of the root system and causing an unstable condition of the tree stand. Contribution deadwood is higher in *A. mangium* Year 4, also caused by windthrow within the stand in the current study. S. S. Lee (2018) discovered that windthrow incidence caused more than 20% mortality, on average, of 5-and-a-half year-olds of *A. mangium*, stands in Sarawak. *Acacia mangium* is recognized for having a shallow root system that spreads and is, therefore, quite vulnerable to windthrow and easily blown down. However, the trees could also fall by the wind more easily, as the root system is not stable because of the damage caused by root-rot disease (S. S. Lee, 2018). The observation was also similar to that reported by Pfeifer et al. (2015), who stated that the higher contribution of deadwood was linked with environmental factors.

Furthermore, there is no standing deadwood recorded in *A. mangium* stand Year 2. It indicates that 2 years-old *A. mangium* stand is the youngest and has a vigorous growth rate. According to Hardiyanto and Wicaksono (2008), the peak nutrient demand and growth of *A. mangium* occurred during the first three years. *Acacia mangium* stand at Year 9 shows lower deadwood carbon content statistically, and no stump deadwood was recorded. It could be attributed to the decomposition of deadwood, which frequently occurs during this period, especially for stumps. Deadwood decomposition in degraded tropical forests is estimated to take six to nine years on average (Chambers et al., 2001; Pfeifer et al., 2015; Rice et al., 2004).

**Litter and Soil Carbon Content**

Litter plays an important role in the plantation and natural forest carbon cycle. According to the current study, the control contributed more litter carbon than *A. mangium* stands in Years 2 and 4 and not Year 9 (Table 1). As expected, the litter carbon in control is higher than *A. mangium* stands due to the contribution of tree biomass and various tree species that provide higher litter production. Other researchers also reported that diverse compositions have higher litter yields in both amount and quality when compared to monocultures, which could enhance tree productivity and carbon sock (Giweta, 2020). The results also show that the lower amount of litter carbon in *A. mangium* in Years 2 and 4 is due to land use change, which exposes litter to a high decomposition rate (K. L. Lee et al., 2015). Moreover, Giweta (2020) stated that the hugely disturbing area produces lower litter production compared to less disturbed areas.

However, higher litter carbon in *A. mangium* stands Year 9 is due to the contribution of false staghorn fern (*Dicranopteris linearis*). These ferocious ferns form dense thickets, contributing to a higher percentage of forest litter on the top surface. In addition, the fern sustains

and suppresses its dominance by covering the area with an extensive root system and slow-decomposing leaves (Shono et al., 2006). The low decomposition rate of *D. linearis* is due to the phenolic compounds found in their leaves (Othman et al., 2020) and the fern species' high lignin compounds (Amatangelo & Vitousek, 2009).

Land use change of forests also contributed to the decline in SCC due to the change in carbon sequestration (K. L. Lee et al., 2015). It is demonstrated by the results, which show that the control has significantly higher SCC than all *A. mangium* stands at both soil depths. It is due to the decomposition of plant debris, such as litter, twigs, brunch, and roots. Therefore, the decline of SCC in the disturbed forest (converted into plantation) is due to the reduction in the amount of plant debris returning into the soil and an increase in the quantity of carbon dioxide released into the atmosphere from the decomposing process of soil organic matter.

Furthermore, the SCC of *A. mangium* stand Year 9 was lower at depths of 0–15 cm and similar at depths of 20–35 cm when compared to Year 4 due to the low decomposition rate of *D. linearis*, even though the highest litter carbon was recorded. While the SCC in Year 2 was lower when compared to Year 4 and litter carbon within these ages. According to K. L. Lee et al. (2015), SCC was influenced by the decomposition of litter, which is affected by the ecological factors of the area and human activity. Year 2 was in the youngest stage of tree growth, with the cumulative canopy openness causing rapid drying out of the litter in sunlight, potentially resulting in a decline in microbial decomposition (Paudel et al., 2015).

While SCC in Year 4 was statistically higher than the other two *A. mangium* stands because of environmental and climate factors, such as soil property, temperature, and humidity, that can affect the decomposition of litter (Giweta, 2020). Year 4 probably has higher litter decomposition due to healthier canopy covers that create a favourable microclimate and are suitable for microbial decomposer populations. Among the four forest stands of this study, SCC was the highest in the surface soil layer (0–15 cm) and indicated a decreasing trend with depth. It can be explained by the fact that it had the highest quantity of SCC, which significantly increased soil organic matter as a primary source of soil carbon and is higher in topsoil (Levan et al., 2020).

### Total Carbon

Total carbon content is in line with all classes of terrestrial carbon pools, where probably a decline in the amount of carbon tends to occur due to land use change. According to Ratnasingam et al. (2016), the conversion of natural forests, especially the secondary or logged-over forests, to plantation forests assists in decreasing the amount of carbon stock in the environment due to the change in carbon-storing capacity. It is also demonstrated by the results of this study, which compares the percentage of total carbon in *A. mangium* stands in Year

2 (26.39%), Year 4 (56.02%), and Year 9 (34.42%) to the control as a benchmark.

Referring to previous studies, such as Vashum and Jayakumar (2012) and Ratnasingam et al. (2016), deadwood contributes only a small amount of carbon to forest carbon stock. However, this study is inconsistent with those in which the contribution of deadwood to total carbon content is higher. This study supported previous research by Pfeifer et al. (2015), who discovered that deadwood stores significant amounts of carbon, accounting for more than 50% of AGB carbon stock in degraded tropical forests in Sabah, Malaysia. While this study also found that total deadwood carbon stored was 59% of AGB carbon. The higher contribution of deadwood in the control and *A. mangium* plantations is due to the condition of the logged-over forest that generates higher deadwood from the reduction in the AGB by the previous logging activities. Logging removed large trees and caused residual logging damage to other live trees (Pfeifer et al., 2015). Consequently, AGB carbon stocks in some of the logged-over forest stands were severely reduced. This condition is exemplified by the fact that the control had higher deadwood carbon (273.30 t/ha) than AGB carbon (104.71 t/ha) in this study (Table 1). Moreover, the existence of various tree species also contributes to the high amount of deadwood with different decomposition rates (Pfeifer et al., 2015).

While *A. mangium* stands also recorded a high amount of deadwood carbon in Year 2 (59.45 t/ha), Year 4 (185.05 t/ha), and Year 9 (37.89 t/ha) when compared to AGB carbon in Year 2 (2.11 t/ha), Year 4 (7.67 t/ha), and Year 9 (29.78 t/ha). However, fallen deadwood recorded a higher contribution in the total deadwood carbon of *A. mangium* Year 4 that assumes influence by environmental factors, such as the higher terrain steepness and the existence of old skid trails from previous logging activities that create unstable conditions for soil and tree stands. It also may be caused by windthrow incidence and insect infestation or disease (S. S. Lee, 2018). These environmental and previous anthropogenic factors contribute to an unstable root system and tree stand, leading to mortality and the conversion of living trees to dead, broken, or downed large woody debris.

## CONCLUSION

The choices of species planted are vital to ensure that the soil can provide an adequate amount of nutrients, as the requirement for nutrients varies from one species to another. *Acacia mangium* is considered one of the best species to be cultivated as it could fix the N by itself, thus lessening the scarcity issue of N in the plantation site. Therefore, the only nutrient-related concern that should be emphasized in planting this tree species is the availability of P and other macronutrients in the soil for the tree's uptake.

Further application of fertilizer would assist in providing more nutrients, especially P, to the soil while at the same time increasing the biomass of the trees. The application of fertilizer should not only be limited to the

young ages of the tree but also the older ones like Year 4 and Year 9 acacia stands. However, further research is needed to study the fertilizer requirement by the different ages of the stands and its application as well as other nutrient availability status in this study area so that better improvement and management can be made. Silvicultural practices also significantly reduce the competition for nutrients among the planted trees. Moreover, thinning practices also would produce more high-quality trees with financial benefits. Slashing would lessen the unwanted wildings while at the same time could provide easy accessibility into the planting area.

*Acacia mangium* plantation also has demonstrated the potential role of carbon sink. Forest areas, either natural or plantation, must be preserved to mitigate the effects of global warming caused by increased carbon emissions in the atmosphere. *Acacia mangium* stands in the current study and recorded significantly lower total terrestrial carbon content compared to the control. The conversion of natural forest to plantation forest reduced carbon content during the early stages of plantation establishment. This study also showed that old plantation stands contained much more total carbon stock than younger ones. It can be explained by the fact that the content of tree biomass carbon has a good relationship with the stem size. At the same time, the stem size increases linearly with the age of the stand. This study also revealed that deadwood is important in determining total terrestrial carbon stock (the control and Year 4).

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# Influence of Irrigation Systems on the Plant Growth and Leaf Ratio Analyses of Rubber (*Hevea brasiliensis*) Seedlings

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## ABSTRACT

The sprinkler irrigation system is the most widely used system for rubber irrigation in a nursery. However, the method is associated with high water loss during irrigation. In view of this, an experiment was conducted to assess the effect of different irrigation systems on the growth dynamics, leaf ratio analyses, water productivity, and water use efficiency of rubber seedlings. The treatments were the irrigation systems; soil + overhead sprinkler (CON), growing media (GM) + drip irrigation (DRP), GM + capillary wick system (WCK), and GM + overhead sprinkler (SPR). Each treatment was replicated three times, and the experiment was laid out in a randomized complete block design. The results showed that the DRP and WCK had significantly ($p<0.05$) higher seedlings' growth parameters by 15–39% than obtained in the SPR and CON. However, the DRP, WCK, and SPR had statistically comparable seedlings' root length and volume by 14–43% higher relative to the CON. Similar trends of plant growth dynamics, such as crop growth rate (CGR), leaf area index (LAI), and leaf ratio analyses, were observed for all treatments. However, the CON had lower CGR and LAI, which could be attributed to the lower water retention of the soil used in the treatment. The DRP and WCK had comparable water productivity with 56–60% higher than the SPR and CON treatments. The sprinkler irrigation treatments (SPR and CON) had 84% lower water use efficiency than the DRP and WCK. The DRP and WCK are the best treatments in this study because their higher water application uniformity led to higher seedlings' growth dynamics

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and water productivity. The sprinkler system had higher water loss due to the lack of application uniformity, leading to lower plant growth than other irrigations. However, the SPR shows the potential to be more cost-effective due to its lower recurrent cost of labor than drip and wick irrigation.

*Keywords*: Drip irrigation, growing media, sprinkler irrigation, water productivity, wick irrigation

## INTRODUCTION

Rubber is produced in over 10.3 million hectares globally, and 93% of its extent comes from the Asian region (Nair, 2021). The increase in rubber production is necessary to face high latex demand worldwide (Nair, 2021). Latex production lies in developing and distributing early maturing seedlings, disease free and vigorous, which could guarantee a high field survival rate (Nabayi, 2016). Rubber seedlings are typically raised in nurseries, where they go through desired manipulations for a high field survival rate which is the basis for high latex yield (Waizah et al., 2011). Therefore, choosing an efficient and uniform water distribution irrigation method that ensures constant moisture in the root zones of rubber seedlings would lead to higher latex yield after field transplanting.

In Malaysia, there are no options for raising rubber seedlings in the nursery other than the conventional soil-polybag system (Nabayi, 2016). In the conventional soil polybag method, labor accounts for more than 80% of the total cost associated with rubber production in the nursery (Nabayi et al., 2020). The use of compost as a soil substitute to raise rubber seedlings has recently been practiced (Salisu et al., 2013). Many potting mixes and root trainers are being introduced to replace the soil-polybag system of seedlings' growth (Nabayi et al., 2018). The introduction of the growing media should result in several advantages, including lighter weight and compact structure that makes it eco-friendly as the container used with the growing media can be recycled, improved root growth, and reduced labor because of its design that makes it relatively easier to handle than polybag. In contrast, using a soil polybag system requires much space because the polybag cannot be recycled (Bunt, 1988).

Water shortages affect cell differentiation and enlargement, which determines plant growth (Cabuslay et al., 2002; Correia et al., 2001). Furthermore, a lack of uniform water distribution around the roots of plants restricts plant growth by inhibiting biochemical and physiological processes, which include photosynthesis, respiration, translocation, nutrients uptake, and carbohydrate metabolism (Bhatt & Rao, 2005; Chaitanya et al., 2003). Therefore, water shortage is the most critical environmental factor affecting crop productivity (Raj et al., 2005). It reduces the amount of water available to plants in the soil, which leads to lower plant growth and yield (da Silva et al., 2013). Several studies (Dey & Vijayakumar, 2005; Sumesh et al., 2011; Thomas et al., 2015) reported that subjecting young rubber seedlings to a prolonged water shortage decrease growth

and physiological attributes, chlorophyll instability, and suppresses photosynthesis.

Water is the most dynamic and limiting environmental factor impacting plant productivity. Warm temperatures, sunlight, nutrients, and water are all required for crops to thrive. Temperature and sunlight are available in various parts of the planet; however, water is deficient (Nabayi, 2016). All plants require an optimum amount of water to grow and a maximum amount for maximum yield (Field & Long, 2018). Sprinkler irrigation, drip irrigation, and capillary wick irrigation are alternative water application technologies that can help optimize water use in agriculture and increase irrigation efficiency (Heydari, 2014). Sprinkler irrigation systems are popular because they are inexpensive to install, but they waste much water from the usual over- and under-watering plants due to their lack of water application uniformity (Nabayi, 2016). Drip irrigation saves water since less water is lost in the application process, and water is delivered directly to the plant (Maya et al., 2014). Another water-saving technique is the capillary wick irrigation system, which uses the capillary action of the wick to slowly deliver water to the plant roots (Bainbridge, 2002). The problem of low crop productivity can be overcome with the aid of a proper irrigation system. Climate change, water scarcity, and population growth have all increased efforts to find new techniques to save water for irrigating crops, the world's largest water user (Field & Long, 2018). Therefore, the objectives of this study were to ascertain (1) the effect of different irrigation systems on the growth dynamics and leaf ratio analyses of rubber seedlings and (2) the effect of different irrigation systems on water productivity and water use efficiency of the rubber seedlings.

## MATERIALS AND METHODS

### Experimental Site

The experiment was conducted in a rain shelter facility (2°59'05.0"N 101°44'00.9"E), Agrobio Complex, Faculty of Agriculture, Universiti Putra Malaysia (UPM), from December 2014 to July 2015. A mini weather station (WatchDog 2000 series, Spectrum Technology Inc., USA) was installed to monitor the environmental conditions in the rain shelter. BX-1 growing media (GM) (Humibox Sdn. Berhad, Malaysia) was used as the potting mix, and Rb900 (Figure 1) root trainer was used as the growing container for overhead sprinkler (SPR), drip (DRP), and capillary wick

*Figure 1*. Rb900 Root trainer

(WCK) treatments based on its design and suitability as previously reported by Nabayi et al. (2016), which allows plant roots to grow downward and prevent spiral growth. An amount of 230 g of the fresh GM was used per root trainer in SPR, WCK, and DRP treatments, while 8 kg of Munchong soil series (Tropeptic Haplorthox) per polybag was used for CON treatment. The soil used in the CON treatment was initially treated with 25 g of powdered Christmas Island Rock Phosphate (Cilibangi, Malaysia) as a starter during transplanting into the polybag system, which was followed by a regular NPK fertilizer (N-P-K- 15-15-15 mixture) (Cilibangi, Malaysia) application as stated in Noordin (2013).

### Treatments and Experimental Design

The treatments were soil + overhead sprinkler (CON), growing media (GM) + drip irrigation (DRP), GM + capillary wick system (WCK), and GM + overhead sprinkler (SPR), which were replicated three times each and laid out in a randomized complete block design. For the GM treatments (DRP, WCK, and SPR), their seedlings were transplanted in the Rb900 tube, while for the CON, the seedlings were transplanted into a polybag. The rubber seedlings were transplanted at one-month-old. One hundred and twenty (120) rubber seedlings were used in the experiment, with 10 seedlings per replication. In addition, the clone RRIM 2000 secured from MARDI (Malaysian Agricultural Research Development Institute) was used in the experiment. A daily supply of 11 mm of water was provided in the DRP, SPR, and CON irrigation systems. Before starting the experiment, the irrigation systems were calibrated to supply 11 mm water equivalent by setting the drip and sprinkler irrigation timers for 3 min 20 secs and 2 mins, respectively, in 24 hours. The basis for choosing 11 mm for the sprinkler and drip irrigation was because the wick system is self-watering irrigation, which was applied 11 mm into the seedlings container in 24 hours.

### Physical and Chemical Analyses of Soil and Growing Media

CNS analyzer (LECO Corp., USA) was used to analyze the total carbon (C), nitrogen (N), and sulphur (S) of the soil and GM. Next, the leaching method was used to determine the cation exchange capacity (CEC) and exchangeable bases (Chapman, 1965), after which the exchangeable bases were assessed using atomic absorption spectrophotometer (AAS) (PerkinElmer, 5100PC, USA). Next, phosphorus (P) was determined using an auto-analyzer (AA) (Quikchem FIA 8000 series, LACHAT Instruments, Canada). Finally, the physical properties of the GM and soil were conducted as outlined in Jones (2001) (Table 1).

### Plant Growth Measurements

A graduated ruler was used to measure the plant height. The girth size of the seedlings was assessed using a vernier caliper. The total leaf area was measured with a leaf area meter equipment (LI-3100C Area meter, Lincoln, USA). Destructive sampling was used to measure plant growth characteristics

Table 1
*Average (±SE) physical and chemical properties of the soil and growing media*

| Physical properties | Soil | Growing media (BX-1) |
|---|---|---|
| BD (g $cm^{-3}$) | 1.43±0.02 | 0.14±0.01 |
| MC (g $g^{-1}$) | 0.21±0.06 | 0.71±0.01 |
| Total Porosity (%) | 46.0±3.10 | 91.0±2.01 |
| SHC (cm $hr^{-1}$) | 8.2±0.20 | 32.0±0.04 |
| Saturation ($m^3$ $m^{-3}$) | 0.56±0.05 | 0.95±0.04 |
| Field capacity ($m^3$ $m^{-3}$) | 0.29±0.02 | 0.31±0.02 |
| Permanent wilting point ($m^3$ $m^{-3}$) | 0.21±0.07 | 0.20±0.01 |
| Particle size analysis | | |
| Sand (%) | 34.54±0.02 | - |
| Silt (%) | 15.23±0.01 | - |
| Clay (%) | 50.21±0.02 | - |
| **Chemical properties** | **Soil** | **Growing media (BX-1)** |
| pH | 4.67±0.30 | 6.40±0.90 |
| EC (dS $m^{-1}$) | 0.04±0.002 | 1.22±0.03 |
| CEC ($cmol_+ kg^{-1}$) | 8.32±0.10 | 63.21±0.40 |
| C (%) | 1.38±0.10 | 34.25±0.20 |
| N (%) | 0.13±0.02 | 1.09±0.20 |
| C: N | 10.6±0.02 | 27.0±0.10 |
| S (%) | 0.03±0.001 | 0.75±0.001 |
| P (ppm) | 8.34±1.02 | 680.57±8.30 |
| K (ppm) | 41.27±3.10 | 1779±13.21 |
| Ca (ppm) | 459.33±4.70 | 6223.67±17.60 |
| Mg (ppm) | 85.47±3.90 | 1709.33±23.70 |
| Na (ppm) | 5.43±0.30 | 17.93±0.92 |

*Note*. BD = Bulk density; CEC = Cation exchange capacity; MC = Moisture content; SHC = Saturated hydraulic conductivity

such as fresh and dry weight and root parameters at harvest (8th month). The plant samples were separated into different parts, i.e., roots, leaves, and shoots, and measured for both fresh and dry weight using a weighing machine (Multitech, GF-3000, Japan). The different parts of the seedlings were placed in a forced draft oven at 72 $^{o}C$ for 48 hours to obtain the dry weights and then measured using a weighing balance. The root analyses were done using EPSON WhinRhizo root scanner (Epson Perfection V700 Photo, Reagent Instrument Inc., Canada).

## Plant Growth and Leaf Ratio Analyses

Hunt (1990) defined the plant growth analysis and leaf ratio parameters. Relative growth rate (RGR) is the efficiency index that explains an increase in plant size per unit area. It is relatively an increase in the plant's total dry weight. Crop growth

rate (CGR) is an index of the productive efficiency of ground area in producing plant dry weight. Net assimilation rate (NAR) is also an index of productive plant efficiency in relation to the total leaf area. The leaf area index (LAI) is the ratio between the total leaf area and the ground area it covers. Leaf weight ratio (LWR) helps explain a plant's productive investment as it deals with relative expenditure on potentially photosynthesizing plant parts. Leaf area ratio (LAR) is the relationship between potentially respiring and potentially synthesizing organs.

Therefore, for the computation of the mean growth analyses of the rubber seedlings grown under different irrigation systems over eight months, the formula by Hunt (1990) was adopted as follows:

Mean RGR ($g\ g^{-1}\ day^{-1}\ month^{-1}$) =

$$= \left(\frac{\log_e W2 - \log_e W1}{t2 - t1}\right) \quad [1]$$

Mean CGR ($g\ m^{-2}\ day^{-1}$) =

$$= \left(\frac{1}{P}\right) \times \left(\frac{W2 - W1}{t2 - t1}\right) \quad [2]$$

Mean LAR ($m^2\ cm^{-2}\ g^{-1}$) =

$$= \left(\frac{\frac{LA1}{W1} + \frac{LA2}{W2}}{2}\right) \quad [3]$$

Mean LWR=

$$= \left(\frac{\frac{Lw1}{W1} + \frac{Lw2}{W2}}{2}\right) \quad [4]$$

Mean LAI=

$$= \left(\frac{\frac{LA1}{P1} + \frac{LA2}{P2}}{2}\right) \quad [5]$$

Mean NAR ($g\ m^{-2}\ day^{-1}\ month^{-1}$) =

$$\left(\frac{(W2 - W1)}{(t2 - t1)} \times \frac{(\log_e LA2 - \log_e LA1)}{(LA2 - LA1)}\right) \quad [6]$$

where $W$ is the total dry plant weight, $L_A$ is the total leaf area, $L_W$ is the whole leaf dry weight, $log_e$ is the natural logarithm, $P$ is the total ground area upon which the crop stands, and $t$ is the time at which the samples were taken.

**Water Productivity and Water Use Efficiency**

Water productivity (WP) and water use efficiency (WUE) were determined based on Heydari's (2014) concept of WP (Equation 7) and WUE (Equation 8).

$$\text{WP}\ (\text{gL}^{-1}) = \frac{Total\ plant\ dry\ weight\ (g)}{Cumulative\ transpiration\ (L)} \quad [7]$$

$$\text{WUE} = \frac{Quantity\ of\ water\ used\ by\ the\ plant\ (L)}{Output\ of\ the\ irrigation\ system\ (L)} \quad [8]$$

The cumulative transpiration of the rubber seedlings was determined using equation 9 (water-balance equation).

$$T = I - (L + E + \Delta\theta) \quad [9]$$

where $T$ is transpiration (mm), $I$ is irrigation (mm), $L$ is leaching (mm), $E$ is evaporation (mm), and $\Delta\theta$ is the change in moisture storage (mm).

**Data Analysis**

All the data collected were analyzed using Minitab (version 20) for Windows

(Pennsylvania State University, USA). Analysis of variance (ANOVA) was used to determine the significant treatment effects of the measured parameters ($p<0.05$). Significant means were separated using Tukey's honestly significant difference (HSD) test at a 5% significance level.

## RESULTS AND DISCUSSION

### Plant Growth of Rubber Seedlings

The treatments did not differ significantly ($p>0.05$) in terms of specific leaf area, plant height, and number of leaves. However, the plant height and the number of leaves increased significantly ($p<0.01$) with time for all treatments. The rubber seedlings under WCK and DRP had significantly ($p<0.01$) higher total fresh weight, total dry weight, and shoot and root dry weights (Figure 2), which differed from the SPR and CON, that had the lowest. The WCK and DRP had a range increase in total fresh and dry weights by 14–26% and 26–36%, respectively, relative to the SPR and CON treatments. Similarly, the WCK and DRP had a range increase in seedlings' shoot and root dry weights by 15–36% and 17–36%, respectively, relative to the SPR and CON treatments. However, despite the growth of

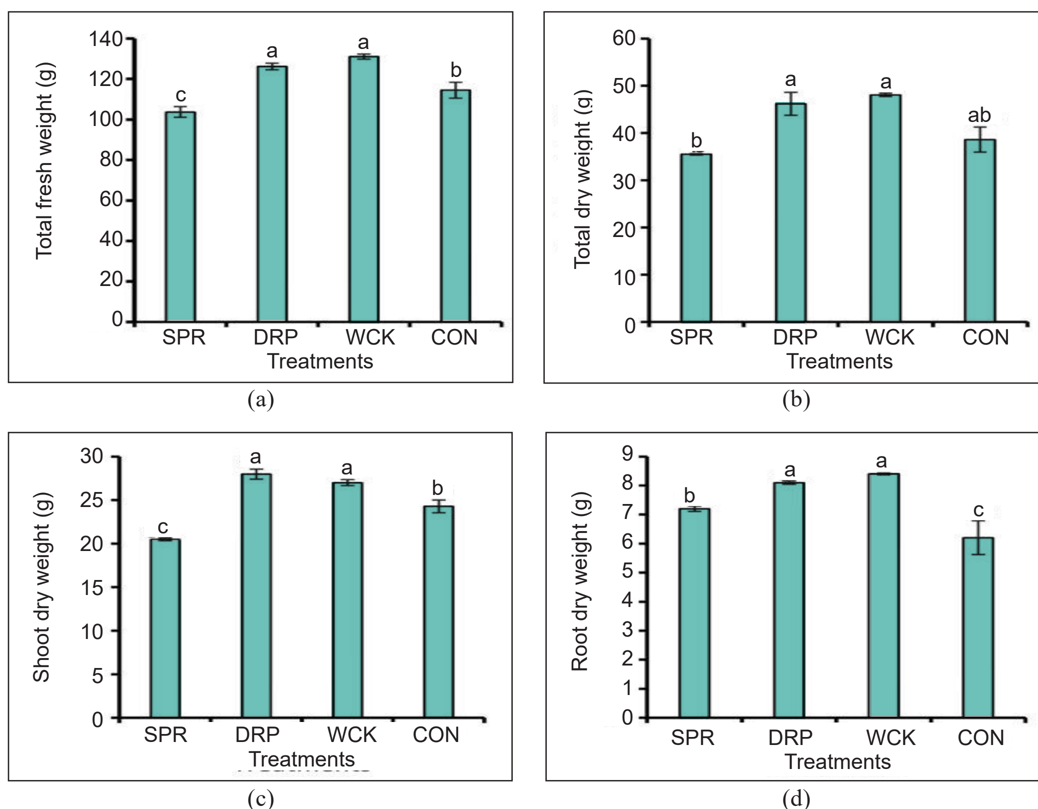


*Figure 2*. Means (± SE) of (a) total fresh weight, (b) total dry weight, (c) shoot dry weight, and (d) root dry weight of rubber seedlings as influenced by different irrigation systems. Means followed by different letter(s) differ significantly ($p<0.05$) from one another using Tukey's test

the statistically comparable seedlings in the SPR and CON treatments, the CON had a 20% higher shoot dry weight than the SPR treatment. In contrast, the SPR had a 13% increase in root dry weight than the CON.

The highest seedlings' total fresh and dry weights, shoot and root dry weights recorded in WCK and DRP systems could be attributed to the efficiency of the irrigation systems to supply the water uniformly in the root zone of the seedlings, as opposed to the SPR and CON that were both irrigated using a sprinkler. Nabayi et al. (2018) reported higher seedlings' growth parameters in drip and wick irrigation systems, which they attributed to the systems' moisture availability, as water is applied slowly into the root zones of the seedlings. Parameters, such as plant water-absorption patterns, are influenced by irrigation systems, which ultimately affect plant growth (Argo & Biernbaum, 1994). Drip irrigation is critical in sustaining crop productivity and minimizing water use in agriculture (El-Hendawy et al., 2008). The lower seedlings' growth parameters obtained in the SPR system and CON could be due to the lack of sufficient moisture because of the non-uniform water distribution of the overhead sprinkler which applies more water outside than inside the growing containers. Westervelt (2003) stated that lack of irrigation uniformity causes water loss, which means more water is needed to supply the required amount.

The WCK had a range increase in leaves dry weight by 20–39% higher than the other treatments (Figure 3). The WCK and DRP had 21–26% higher total leaf area than the SPR and CON. Water and sunlight determine dry matter accumulation, which could be the reason for the higher leaves and root dry weight in the WCK and DRP treatments. Aydinsakir et al. (2013) reported higher total plant dry weight in 100% soil water application than in the 75%, 50%, and 25% applications. Heydari (2014) claimed that drip irrigation is the most effective system than sprinkler because of its excellent water use efficiency. Water application efficiency in drip systems approaches 100%, with 30–50% water savings compared to other irrigation methods (Ozsan et al., 1983). Rivera et al. (2009) reported a decrease in yield and biomass components of corn due to the lack of sufficient moisture in the crop root zone. Many studies have found irrigation types, regardless of source and nutrient solution, to impact nursery seedlings' growth (Argo & Biernbaum, 1994). The lower seedlings' girth size obtained in the SPR and CON could be attributed to the lack of sufficient moisture distribution into the seedlings growing container, which agrees with Çakir (2004), who reported a decrease in corn plant girth size due to a lack of moisture uniformity around the root zone of the plant.

At harvest, the seedlings in CON had the highest shoot-to-root ratio of 5.26, which did not differ significantly ($p>0.05$) from those raised in WCK (4.73) and DRP (4.7) systems (Figure 3d). However, the three systems differed significantly ($p<0.05$) from the SPR system, which had the lowest seedlings' shoot-to-root ratio with 3.92. An increase in the shoot-

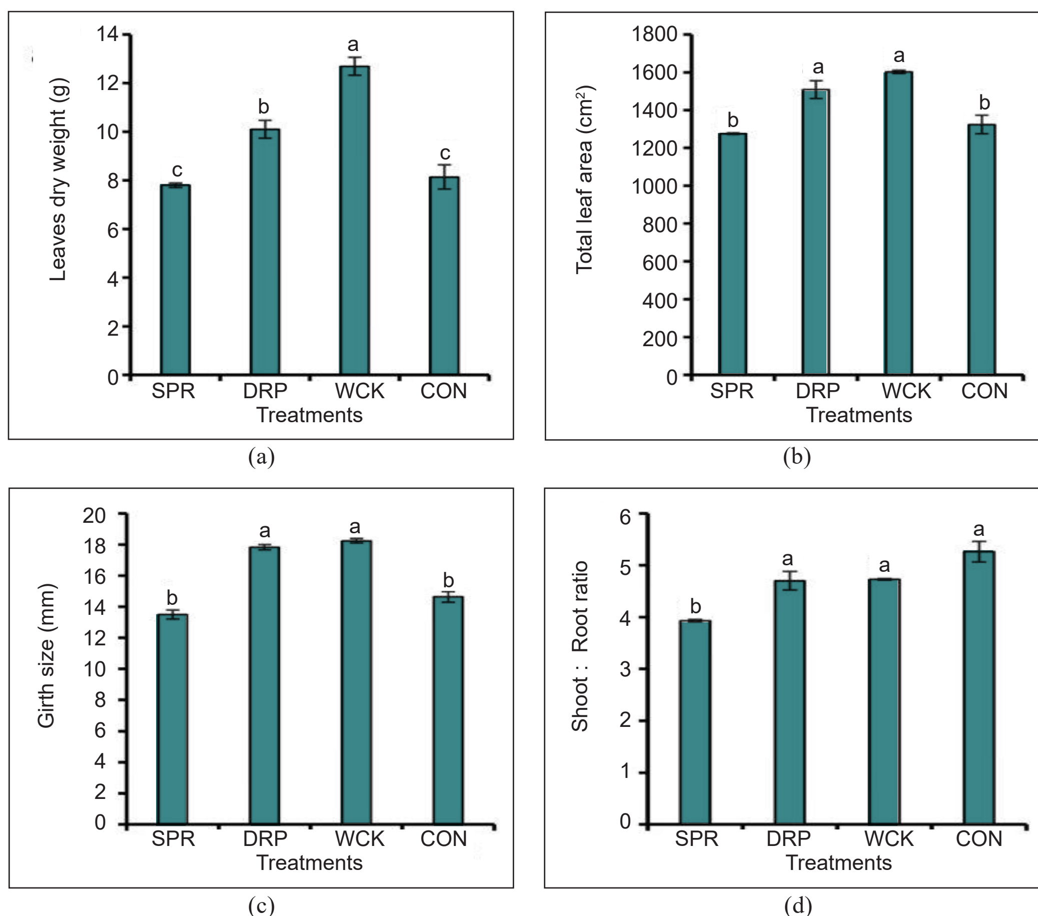


*Figure 3*. Means (± SE) of (a) leaves dry weight, (b) total leaf area, (c) shoot to root ratio, and (d) girth size of rubber seedlings as influenced by different irrigation systems. Means followed by a different letter(s) differ significantly ($p<0.05$) from one another using Tukey's test

to-root ratio signifies a higher priority for photosynthate accumulation in shoots than roots. Conversely, if shoot-to-root ratios decrease with time, roots have preferential utilization of photosynthate under the present plant growing conditions (dos Santos et al., 2007; Franco et al., 2006). The lower shoot-to-root ratios in the SPR was because of the moisture shortages between irrigation intervals in the system as the system applied more water outside the opening area of the seedlings' container (Abba et al., 2015; Nabayi et al., 2018). Dos Santos et al. (2007) explained that a higher shoot-to-root ratio indicates a lack of moisture deficit. Furthermore, Mackay and Barber (1985) stated that water and N deficiencies are the most limiting factors for shoot growth.

## Root Growth Parameters of Rubber Seedlings

The DRP, WCK, and SPR had a range increase in seedlings' root length and

root volume by 32–43% and 14–34%, respectively, which differed significantly ($p$<0.05) from those raised in CON treatment (Figure 4). However, the highest root surface area was observed in DRP and WCK treatments with 12–22% higher than the SPR. The result showed that GM treatments (SPR, DRP, and WCK) had better root lengths than the CON, which could be attributed to the influence of the Rb900 root trainer because of its design. The Rb900 encourages the roots to grow vertically without restricting the roots (Nabayi et al., 2020), unlike in the CON, where the vertical growth is restricted by the polybag, which in turn could affect the water and nutrients uptake. Vertical root growth leads to a better root system with intact root tips and fewer or no circling roots (Salisu et al., 2018). Higher root growth parameters in the SPR, DRP, and WCK could also be due to the light weight of the media, which encouraged the continuous growth of the root as compared to the soil. In addition, containers with internal ridges

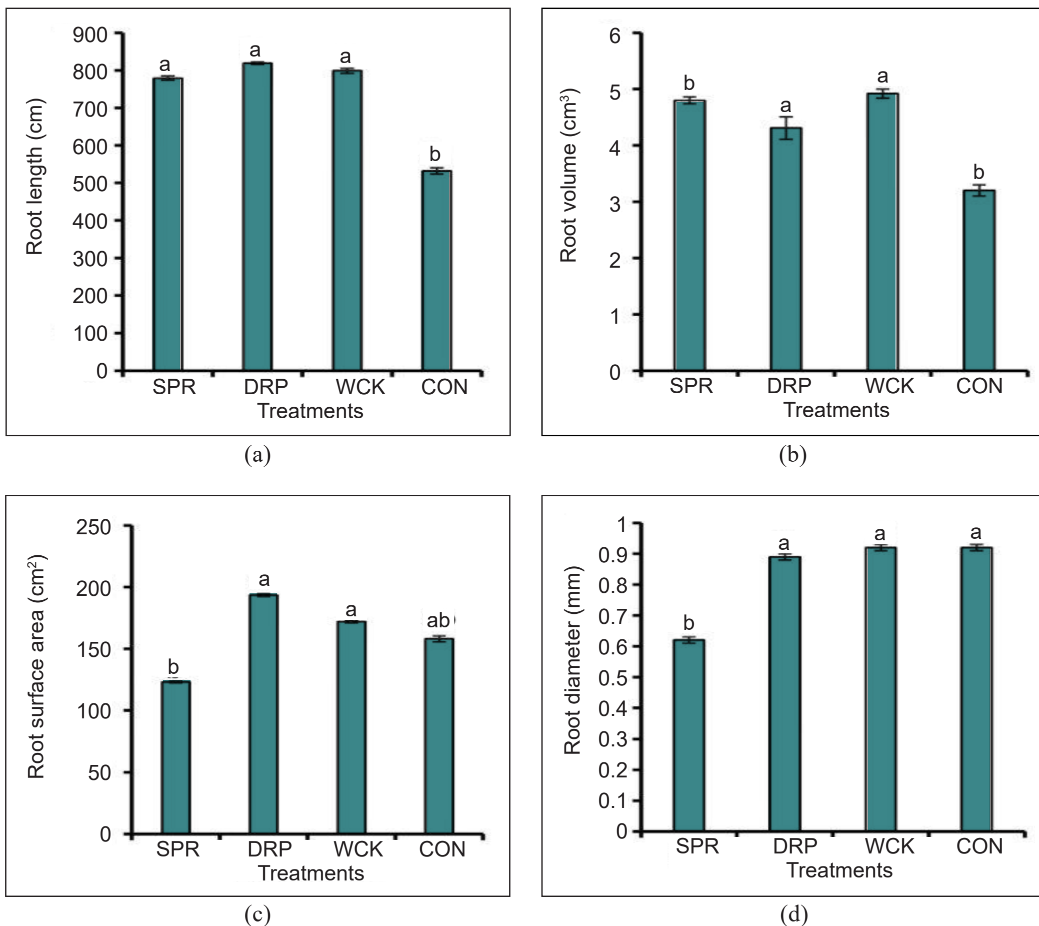


*Figure 4*. Means (± SE) of (a) root length, (b) root volume, (c) root surface area, and (d) root diameter of rubber seedlings as influenced by different irrigation systems. Means followed by a different letter(s) differ significantly ($p$<0.05) from one another using Tukey's test

were designed to limit root circling in container-grown seedlings (Stromberger, 2002), as root cycling could lead to stunted plant growth. Root length and surface area are important indicators for a potential uptake of water and nutrients.

The growing media have greater nutrient availability and can store more water than the soil under the CON treatment. Choosing a proper growing medium is one of the vital considerations in nursery plant production (Bunt, 1988). Generally, the root morphology of the seedlings raised in root trainers differs from those grown in the polybags (Salisu et al., 2018). The vertical root growth restriction of seedings in CON led to the production of thicker roots in the system (0.92 mm) compared with other treatments. Root restrictions can lead to a reduction in primary roots and an increase in lateral roots, which could all result from the type of container used (Salisu et al., 2018). When moisture is available, root restriction can mimic soil moisture stress (Krizek et al., 1985).

### Growth Dynamics and Leaf Ratios Analyses of Rubber Seedlings

The RGR decreased with time, irrespective of the treatments, as the highest and lowest were recorded in the experiment's first and eighth months (Figure 5). However, the NAR increased in all treatments until the third month and remained constant until the eighth month. On the other hand, the LAR and LWR exhibited a similar pattern which increased initially and then declined as time progressed, irrespective of the treatments. Leaf area index (LAI) and CGR trends increased from month 1 to month 8, with higher LAI and CGR in the SPR, DRP, and WCK while the lowest in the CON throughout the months.

Higher RGR in the first month could indicate an efficient utilization of the dry matter by the photosynthetic organs (leaf and stem). The decrease in RGR with time could be associated with the higher leaf defoliation because of the temperature rise (Table 2). Higher NAR was mostly observed in DRP, which indicated its effectiveness toward higher leaves dry weight production, which agrees with our earlier findings (Nabayi et al., 2018). The mean RGR and NAR trends agree with Vijayakumar et al. (1998), who stated that enhanced plant growth dynamics parameters are linked to plant water availability. The WCK had the highest LWR in the eighth month due to the treatment's significantly higher leaves dry weight in the month (Figure 3a). The highest LAI and CGR in the DRP, SPR, and WCK were because of their significantly ($p<0.05$) higher total leaf area (Figure 3b), and it could also be due to the smaller opening area of the growing containers used being the two factors that determine LAI. The increase in LAI with time agrees with Wolf and Carson (1973), who reported that higher LAI in plants indicates plant capability in dry matter accumulation in the non-photosynthetic plant parts (i.e., roots). Similarly, the CGR, LAI, and NAR results observed in this study agree with the trends reported by Hunt (1990), who stated that a decrease in NAR leads to an increase in CGR and LAI of a crop.

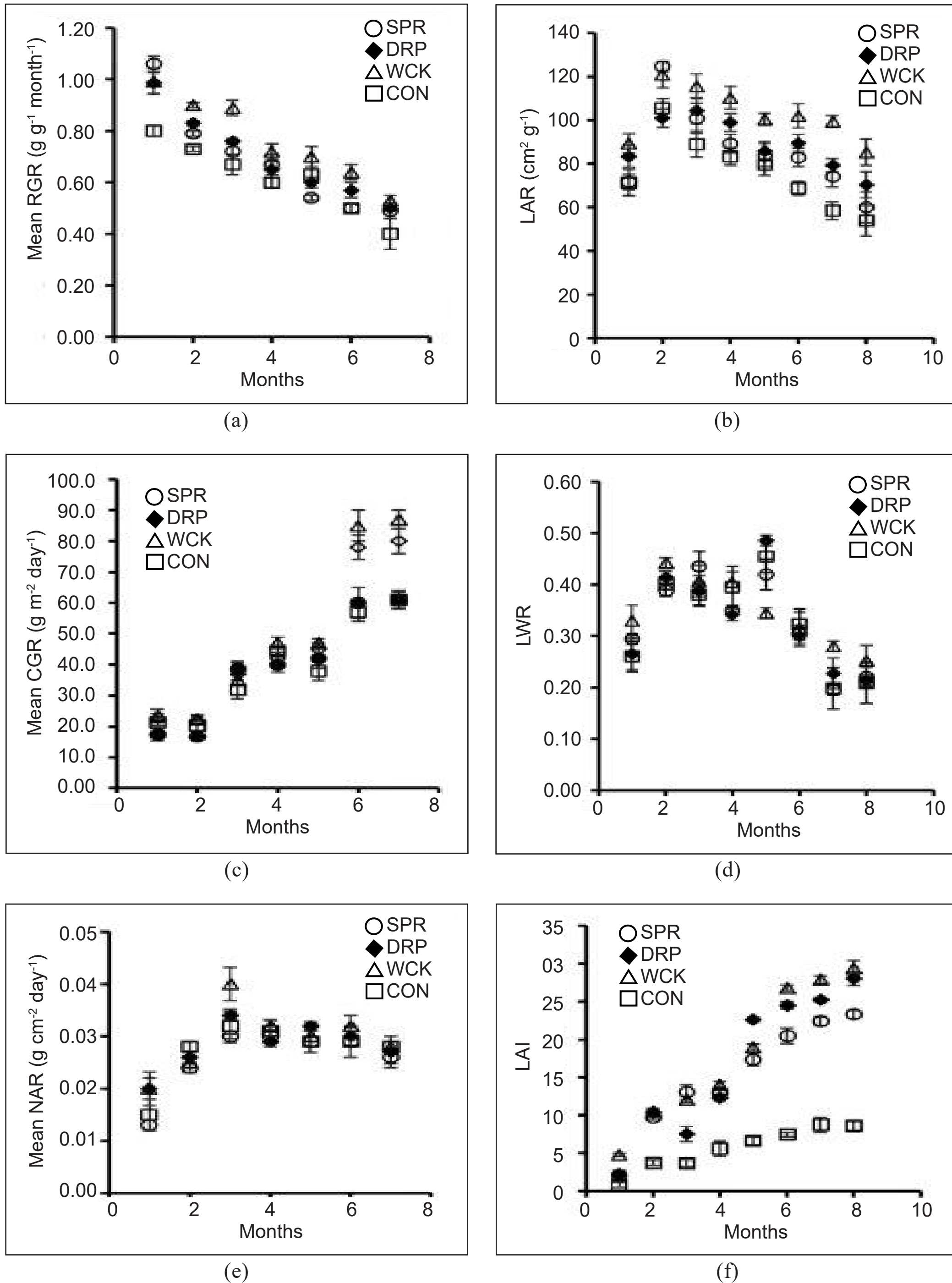


*Figure 5*. Means of (a) RGR, (b) LAR, (c) CGR, (d) LWR, (e) NAR, and (f) LAI of rubber seedlings as influenced by different irrigation systems in 8 months period
*Note*. RGR = Relative growth rate; CGR = Crop growth rate; NAR = Net assimilation rate; LAR = Leaf area ratio; LWR = Leaf weight ratio; LAI = Leaf area index

Table 2
*Means of environmental factors in the rain shelter under which the seedlings grew. Values in brackets are the minimum to maximum recorded range*

| Month | Temperature ($^{o}$C) | Relative humidity (%) | Solar radiation (MJ $m^{-2}$ $day^{-1}$) |
|---|---|---|---|
| Dec | 26.5 (22–35) | 82.5 (36–100) | 2.2 |
| Jan | 26.9 (21–33) | 71.6 (25–98) | 2.9 |
| Feb | 27.8 (20–36) | 64.5 (24–95) | 3.2 |
| Mar | 27.7 (22–37) | 71.8 (29–97) | 3.3 |
| Apr | 27.9 (23–37) | 74.3 (30–99) | 3.4 |
| May | 28.0 (23–36) | 73.8 (38–97) | 2.9 |
| Jun | 27.9 (23–35) | 73.4 (27–95) | 2.6 |
| Jul | 29.8 (23–37) | 83.4 (28–95) | 2.7 |

### Water Productivity and Water Use Efficiency

The seedlings raised in DRP and WCK had statistically comparable ($p$>0.05) WP, but they differed significantly ($p$<0.05) in terms of WUE (Table 3). The seedlings raised in WCK and DRP had a range increase in WP by 56-60% higher than the SPR and CON. However, higher plant WUE was observed in WCK, which differed significantly ($p$<0.05) from other treatments. The seedlings grown in WCK recorded 19% higher WUE than those grown in DRP. On the other hand, both the WCK and DRP treatments had more than 100% higher WUE than the SPR and CON. The significantly lower WP and WUE in SPR and CON seedlings could be attributed to overhead sprinkler irrigation in both treatments. Water productivity is the amount of biomass produced per unit amount consumed. Higher WP of seedlings in DRP and WCK could also be due to lower leaching of N and K in the treatments: the higher the number of nutrients removed, the lower the available nutrients for plant use. The N and K are the two most important elements required by a rubber plant for proper growth and development (Noordin, 2013). The result agrees with Teh et al. (2015), who reported higher WP in water spinach using a wick irrigation system. Higher water use efficiency indicates higher water utilization in relation to the quantity of water supplied. The WUE is one of the most important parameters for determining optimal water management practices (Kharrou et al., 2011). The lowest WUE of seedlings in SPR treatment was due to the overhead sprinkler's lack of uniformity and

Table 3
*Means (±SE) of water productivity and water use efficiency of different irrigation systems at month 8*

| Treatments | WP (g $L^{-1}$) | WUE (L $L^{-1}$) |
|---|---|---|
| DRP | 26.0 ± 1.21a | 0.64 ± 0.03b |
| WCK | 27.1 ± 0.97a | 0.76 ± 0.05a |
| SPR | 17.3 ± 0.27b | 0.11 ± 0.01b |
| CON | 16.9 ± 0.82b | 0.10 ± 0.01b |

*Note*. WP = Water productivity; WUE = Water use efficiency; DRP = Drip irrigation; WCK = Wick irrigation; SPR = Sprinkler irrigation; CON = Control. Means with the same letter within the same column did not differ significantly from one another using Tukey at a 5% level

efficiency, which ended up applying more water outside the plant containers.

To supply 11 mm into the seedlings' containers in SPR and CON treatments using a sprinkler, the system lost about 100 L in the process due to the container size, leaf interception, and lack of uniformity of the overhead sprinkler. Therefore, the sprinkler system lost up to 91%, unlike in the DRP and WCK, where water is only lost through leaching and evaporation from the seedlings' container. Bryant and Yeager (2002) reported that wick irrigation compared with overhead irrigation reduces cumulative irrigation volume by 86% without sacrificing plant growth. In addition, the drip and wick saved more than 90% of water during application relative to the overhead sprinkler irrigation. Therefore, the higher application uniformity, efficiency, and minimal water loss during application in the DRP and WCK led to the higher WP and WUE in their seedlings relative to the seedlings raised using overhead sprinklers as in SPR and CON treatments.

In this study, the sprinkler had a higher initial cost than the drip and wick irrigation systems by 25% and 92%, respectively (Table 4). Furthermore, including the cost of water would make the sprinkler irrigation system more expensive, particularly in water scarcity areas where water is costly. However, sprinklers are preferable for industries, where rubber seedlings are produced in bulk. It would be more cost-efficient because the uniformity of the irrigation system would increase with a higher number of seedlings to irrigate. In addition, the cost of using sprinklers would reduce significantly with time as opposed

Table 4
*Cost of components used for the irrigation systems installation as of 6 Jan 2016*

| Components | Quantity | Unit price (RM) | Total price (RM)[1] |
|---|---|---|---|
| *Overhead sprinkler* | | | |
| Irrigation kits | 1 | 86.8 | 86.8 |
| Timer | 1 | 45.9 | 45.9 |
| Cost of installation | | 60 | 60 |
| Total cost | | | 192.7 |
| *Drip* | | | |
| Irrigation kits | 1 | 47.9 | 47.9 |
| Timer | 1 | 45.9 | 45.9 |
| Cost of installation | | 60 | 60 |
| Total cost | | | 153.8 |
| *Wick* | | | |
| Wick | 1 roll | 27.6 | 27.6 |
| PVC tube | 6 feet | 5.5 | 33 |
| Cost of preparation | | 40 | 40 |
| Total cost | | | 100.6 |

*Note*. [1]RM1 is approximate USD0.24

to drip and wick irrigation, which have a recurrent labor cost. Although the overhead sprinkler had a higher initial cost in this study, which could be compounded in areas with a higher cost of water, for an extended study period, i.e., more than a year, the use of the sprinkler shows the potential to be more cost-effective due to its lower recurrent labor cost.

## CONCLUSION

Sprinkler irrigation system as the conventional system of rubber irrigation in the nursery, is associated with non-uniform water distribution, which impacts the growth dynamics, leaf ratio analyses, water productivity, and use efficiency of the rubber seedlings. Higher fresh and dry weight, total leaf area, girth size, and the shoot-to-root ratio of the seedlings at the end of the experiment were recorded in the DRP and WCK irrigation by 20–30% higher relative to the SPR and CON. It was mainly because of the treatments' uniform moisture distribution and availability compared to the overhead sprinkler. The DRP and WCK had 56–60% higher seedlings' water productivity than those grown in SPR and CON treatments. On the other hand, the use of overhead sprinkler irrigation in SPR and CON had 90% higher water lost during application due to the leaf interception and lack of uniformity that led to high water application outside the seedlings' containers as compared to DRP and WCK that apply water slowly into the vicinity of the seedlings' root. All the treatments followed similar trends for the leaf ratio analyses; however, the CON had significantly lower leaf ratio analyses than the other treatments. Therefore, the study recommends using water-saving irrigation systems (DRP and WCK) for raising rubber seedlings in the nursery in small-scale production. However, for large-scale production, the use of overhead sprinkler irrigation shows the potential to be more cost-effective because of the lower recurrent labor cost involved in the system compared to drip and wick irrigation systems.

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## DECLARATION OF CONFLICT OF INTERESTS

The authors have no conflict of interest.

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# Screening of West Nile Virus, Herpesvirus, and Parvovirus in *Rattus* spp. in Klang Valley, Malaysia

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## ABSTRACT

Free-roaming and scavenging lifestyles of *Rattus* spp. in densely populated urban areas expose them to multiple pathogens that facilitate the transmission of infection to the human population more rapidly, raising public health concerns. There is limited information on the status of rat susceptibility to virus infection, particularly West Nile virus (WNV), herpesvirus, and parvovirus, to prepare for emerging zoonosis. A total of 23 (n = 23) blood samples collected from *Rattus* spp. in the wet market areas of Klang Valley, Malaysia, were subjected to molecular assay using a one-step reverse transcription-polymerase chain reaction (RT-PCR) to detect the highly conserved region of the WNV capsid and pre-membrane protein via nested polymerase chain reaction (PCR) assay targeting highly conserved amino acid motifs within the herpesviral DNA-directed DNA polymerase gene (DPOL) and polymerase chain reaction (PCR) assay targeting the parvovirus non-structural (NS) protein. As a result, 4 out of 23 (17.39%) rats were positive for herpesvirus DNA, but none were positive for WNV RNA and parvovirus DNA. The positive PCR amplicons of herpesvirus DNA were subjected to partial DNA sequencing analysis, 100% identical to *Acomys* herpesvirus SVMS 226,222 from *Betaherpesvirinae*, which is highly

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suggestive of rat cytomegalovirus (RCMV). This study has successfully demonstrated the presence of RCMV from *Rattus* spp. in the Klang Valley. The RCMV potentially crosses species barriers and establishes infection, raising public health concerns. The non-viraemic state of WNV or parvovirus infection, low sample size, and limited niche distribution emphasise the need for the expansion of this study in the future.

*Keywords*: Herpesvirus, Klang Valley, parvovirus, *Rattus* spp., virus, West Nile virus

## INTRODUCTION

Rats, members of the rodent family Muridae, are extremely successful and dominant species worldwide due to their ability to adapt to various environments. As a result, they become the most destructive agricultural pests and vectors for several zoonotic diseases worldwide (Chakma et al., 2018). Furthermore, due to the physiological similarity between rats and humans, their huge diversity, and the fact that some species of rats have adapted to living in close contact with humans, rats play a crucial role as reservoirs and vectors for zoonotic pathogens (Belmain, 2006).

Several countries have reported the seropositivity of *Rattus* spp. to WNV with a low viraemic level. A study in Slidell, Louisiana, showed that WNV seroprevalence in *Rattus* spp. is minimal, at around 5.6% (Dietrich et al., 2005). Black rats (*Rattus rattus*) and house mice (*Mus musculus*), two peri-domestic species of wild rats in Merida, Mexico, had reactive antibodies to flaviviruses, but none was positive for WNV antibodies (Cigarroa-Toledo et al., 2016).

The *Herpesviridae* family consists of three subfamilies: *Alphabetaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. *Rattus* spp. could harbour these herpesviruses before establishing lifelong latent infections in the host. However, they could be reactivated at some point, causing the infectious virus to be transmitted to the other hosts by mucosal contact or droplet infection (Maclachlan & Dubovi, 2017). The possibility of other types of herpesviruses potentially harboured by *Rattus* spp. raises public health concerns due to the potential transmission of infectious diseases to humans.

Among the parvovirus serotypes known to infect the *Rattus* spp. include Kilham's rat virus (KRV), Toolan's H-1 virus, and rat parvovirus (RPV; Maclachlan & Dubovi, 2017). Most clinical signs in rats are derived from the KRV (Kohn & Clifford, 2002). Parvovirus is species-specific, hence, the virus could only spread within the rat population, where the transmission may occur via close or direct contact with the infected rats, through contaminated fomites, and shed in the urine, faeces, milk, and nasal secretions (Macy Jr. & Compton, 2020).

Rats trapped in densely populated urban areas of Klang Valley are exposed to multiple zoonotic pathogens that could be the source of infection in humans (Mohd-Qawiem et al., 2022). It became a cause of concern to public health due to its proximity to humans. Besides, rats could harm laboratory

animal colony biosecurity risk due to inadvertent transmission, possibly causing interference in research (Ain-Fatin et al., 2020). Information on the susceptibility of rats to zoonotic or non-zoonotic virus infection, particularly in WNV, herpesvirus, and parvovirus, are scarce. Therefore, unravelling the information may aid in long-term *in vivo* biosecurity besides preventing potential zoonotic infection. This study aims to screen viruses of veterinary and medical importance in *Rattus* spp. from Klang Valley to provide a current overview of the status of the viruses in Malaysia.

## MATERIALS AND METHODS

### Ethical Approval

This study was approved by the UPM Institutional Animal Care and Usage Committee (IACUC), with an approval code of UPM/IACUC/AUP-U017/2019.

### Sampling

The rats were trapped in selected wet market areas of Klang Valley. The trapped rats (n = 23) were brought to the Post Mortem Laboratory, Faculty of Veterinary Medicine (FPV), UPM, for further examinations. First, the animals were anaesthetised with diethyl ether (Fisher Scientific, USA) at 100 mg/mL for blood collection by intracardiac puncture, followed by terminal exsanguination for euthanasia (Mohd-Qawiem et al., 2022). Then, the blood samples were processed to obtain serum in the Laboratory of Veterinary Virology, FPV. The serum samples were centrifuged at 4,000 × *g* for 10 minutes and transferred to 1.5 mL tubes (Eppendorf, Germany) before storage in the −80°C freezer (SANYO Ultra Low, Japan) until further use. The species, age, and sex of the rats are recorded in Table 1.

### Molecular Analysis

**Nucleic Acid Extraction.** Nucleic acid (RNA and DNA) extraction was performed from the serum samples using TRIsure™ (Bioline, United Kingdom). Extracted RNA was used for detecting WNV, while extracted DNA was used for detecting herpesvirus and parvovirus. RNA and DNA purity and concentration were determined using the BioPhotometer (Eppendorf, Germany) at the absorbance value of 260/280 (Eppendorf, Germany). A value of 1.8 to 2.0 indicates a good purity of nucleic acid.

**Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for Detection of WNV.** Reverse transcriptase polymerase

Table 1
*Number of rats captured relative to the species, sex, and age*

| Rat species | Sex | | Age | |
|---|---|---|---|---|
| | Male | Female | Adult | Juvenile |
| *Rattus rattus* | 8 | 9 | 9 | 8 |
| *Rattus norvegicus* | 4 | 2 | 3 | 3 |
| Total | 23 | | | |

chain reaction (RT-PCR) was performed using primers targeting the highly conserved region between the capsid (C) and pre-membrane (PrM) of WNV (Table 2). A synthetic plasmid was used as the positive control with the addition of a 20 µL master mix (Bioline, United Kingdom). One-step RT-PCR was performed using MyTaq™ One-Step RT-PCR (Bioline, United Kingdom) in a 25 µL reaction, as described previously (Ain-Najwa et al., 2020).

**Polymerase Chain Reaction (PCR) for Detection of Herpesvirus and Parvovirus.** Ready-to-use MyTaq™ Red Mix (Bioline, United Kingdom) was used by preparing a master mix reaction of 7.5 µL of $ddH_2O$, 12.5 µL of MyTaq™ Red Mix, and 1.0 µL each of the forward and reverse primers. Table 2 lists the primer sequences that target the conserved amino acid motifs within the herpesviral DNA-directed DNA polymerase gene and parvovirus non-structural (NS) protein. The PCR tube was then placed in the PCR machine (Eppendorf, Germany) for DNA amplification. The PCR protocols used for herpesvirus and parvovirus include the initialising step at 95 ℃ for 1 minute, denaturation step, 35 PCR cycles of 95 ℃ for 15 seconds, followed by annealing with temperatures at 46 ℃ and 50 ℃ for 15 seconds, and lastly the elongation step at 72 ℃ for 10 seconds.

**Gel Electrophoresis.** Gel electrophoresis was performed to separate the PCR products using 1.5% gel prepared by mixing 1.5 g of agarose gel powder (1st BASE Pte.

Table 2
*Forward and reverse primers used in RT-PCR and PCR analysis for the detection of West Nile virus, herpesvirus, and parvovirus*

| Virus | PCR reaction | Primer | Primer sequence (5’ - 3’) | Product size (bp) | References |
|---|---|---|---|---|---|
| West Nile virus (WNV) | | F | 5’-CCAATACGTTTCGT GTTGG-3’ | 470 | Ain-Najwa et al. (2020) |
| | | R | 5’-ATGTCTTCAGGGTC ATTTCC-3’ | | |
| Herpes virus (nested PCR) | Primary PCR Primer | F | 5’-GAYTTYGCNAGYYT NTAYCC-3’ | 480 | Moureau et al. (2007) |
| | | F | 5’-TCCTGGACAAGCAG CARNYSGCNMTNAA-3’ | | |
| | | R | 5’-GTCTTGCTCACCAG NTCNACNCCYTT-3’ | | |
| | Secondary PCR Primer | F | 5’-TGTAACTCGGTGTA YGGNTTYACNGGNGT-3’ | 215 - 315 | |
| | | R | 5’-CACAGAGTCCGTRT CNCCRTADAT-3’ | | |
| Parvovirus | | F | 5’-AGCACAGGCAGTTG GTAATGTTG-3’ | 1,439 | |
| | | R | 5’-ATAACTCCCAGTAG AAACGCC-3’ | | |

*Note*. F = Forward; R = Reverse

Ltd., Singapore) in 100 mL × TAE (Tris-acetate-EDTA) buffer solution and 5 µL of Red Safe™ (Red Safe™, South Korea). The gel electrophoresis was set at 90 V and 350 mA for 40 minutes. The DNA fragments were then observed under the UV light transilluminator (Syngene, United Kingdom).

**Partial DNA Sequencing.** Unpurified positive PCR products were subjected to DNA sequencing by Apical Scientific Sdn. Bhd. First, they were sequenced with the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) and ethanol precipitation according to the manufacturer's recommendations under an ABI Prism 3130 Fluorescent DNA Analyser (Applied Biosystems, USA). The nucleotide sequences were then aligned using the BioEdit software (version 7.2). Finally, the query sequences obtained were screened against the GenBank nucleotide database using the MegaBlast search function of the National Centre for Biotechnology Information (NCBI) to compare the query sequences and obtain identical nucleotide sequences.

**Phylogenetic Analysis.** The phylogenetic tree was constructed by the maximum likelihood method using the Kimura2 parameter model with 1,000 bootstrap replicates. The analysis comprised 28 reference strains of herpesviruses, targeting the DNA polymerase (DPOL) gene and two outgroups from WNV and parvovirus, shown in Table 3.

Table 3
*List of reference strains used in phylogenetic analysis*

| Herpesvirus | | | | | |
|---|---|---|---|---|---|
| No. | Accession no. | Country | Strain | Year | Isolation source |
| 1 | MF615317 | Brazil | Alpha | 2012 | *Homo sapiens* (Human) |
| 2 | MF615329 | Brazil | Alpha | 2012 | *Homo sapiens* (Human) |
| 3 | EF125072 | Germany | Beta | 2006 | *Rattus tiomanicus* (Malayan wood rat) |
| 4 | HQ587046 | United Kingdom | Unclassified | 2010 | *Acomys dimidiatus* (Eastern spiny mouse) |
| 5 | EF125070 | Thailand | Beta | 2006 | *Rattus rattus* (House rat) |
| 6 | HQ587047 | United Kingdom | Unclassified | 2010 | *Dipodillus dasyurus* (Wagner's gerbil) |
| 7 | AB517983 | Japan | Beta | 2009 | *Miniopterus fuliginosus* (Bat) |
| 8 | HM060767 | USA | Gamma | 2010 | *Loxodonta africana* (Elephant) |
| 9 | NC001826 | USA | Gamma | 1997 | Murid |
| 10 | KY398049 | Congo | Unclassified | 2021 | Wild rodent |
| 11 | KY398052 | Congo | Unclassified | 2010 | Rodent |
| 12 | HQ221963 | Peru | Gamma | 1996 | *Oligoryzomys* (Pygmy rice rat) |
| 13 | MH257598 | Costa Rica | Unclassified | 2011 | *Oligoryzomys vegetus* (Sprightly pygmy rice rat) |
| 14 | MZ934650 | United Kingdom | Unclassified | 2021 | *Myodes glareolus* (Bank vole) |

Table 3 *(continue)*

| No. | Accession no. | Country | Strain | Year | Isolation source |
|---|---|---|---|---|---|
| 15 | KU529537 | France | Alpha | 2008 | *Homo sapiens* (Human) |
| 16 | KM507472 | USA | Alpha | 2012 | *Terrapene Carolina* (Common box turtle) |
| 17 | AF031809 | USA | Gamma | 1997 | *Alcelaphine* (Wildebeest) |
| 18 | AF031812 | USA | Gamma | 1999 | Ovine |
| 19 | AF031811 | USA | Gamma | 1998 | Bovine |
| 20 | AF031810 | USA | Alpha | 1997 | Bovine |
| 21 | MH084656 | Slovenia | Unclassified | 2015 | Ural owl |
| 22 | AF275657 | United Kingdom | Gamma | 2000 | *Meles meles* (The Eurasian badger) |
| 23 | AF159038 | USA | Gamma | 2000 | *Macaca fascicularis* (Long-tailed macaque) |
| 24 | GQ222415 | USA | Alpha | 2007 | *Testudinidae* (Tortoise) |
| 25 | LC008326 | Philippines | Unclassified | 2012 | *Ptenochirus jagori* (Greater musky fruit bat) |
| 26 | AY236869 | USA | Unclassified | 2003 | *Iguanid* (Green iguana) |
| 27 | AF118401 | Germany | Gamma | 1999 | Porcine |
| 28 | KF155695 | Congo | Beta | 2010 | *Mus musculus* (House mouse) |
| 29 | From this study | Malaysia | Beta | 2021 | *Rattus* spp. |
| 30 | From this study | Malaysia | Beta | 2021 | *Rattus* spp. |
| 31 | From this study | Malaysia | Beta | 2021 | *Rattus* spp. |
| 32 | From this study | Malaysia | Beta | 2021 | *Rattus* spp. |
| Outgroup | | | | | |
| No. | Accession no. | Country | Virus | Year | Isolation Source |
| 1 | KJ131503 | Senegal | WNV | 1993 | Rodent |
| 2 | HM989956 | China | Parvovirus | 2010 | *Rattus norvegicus* |

## RESULTS

### Molecular Detection of WNV, Herpesvirus, and Parvovirus

Based on the RT-PCR analysis of the wild rat sera, all 23 rats are negative for WNV RNA, as no band is observed at the 470-base pair (bp) compared to the positive control (Figure 1A). However, the nested PCR analysis of herpesvirus demonstrated that 4 (3 *R. rattus* and 1 *R. norvegicus*) out of 23 (4/23; 17.39%) rats are positive for herpesvirus DNA (Figure 1B). No parvovirus DNA is detected in the PCR analysis, as shown in Figure 1C.

### Sequencing and Phylogenetic Analysis

Based on the sequence comparison with the published sequences from the GenBank database, the current isolates revealed 100% similarity to *Acomys* herpesvirus SVMS 226,222 derived from the *Betaherpesvirinae* subfamily for all positive samples of herpesvirus DNA (Figure 2). The phylogenetic analysis comprises 28 reference strains of herpesviruses targeting the DPOL gene and two outgroups from WNV and parvovirus. The sequences are highlighted based

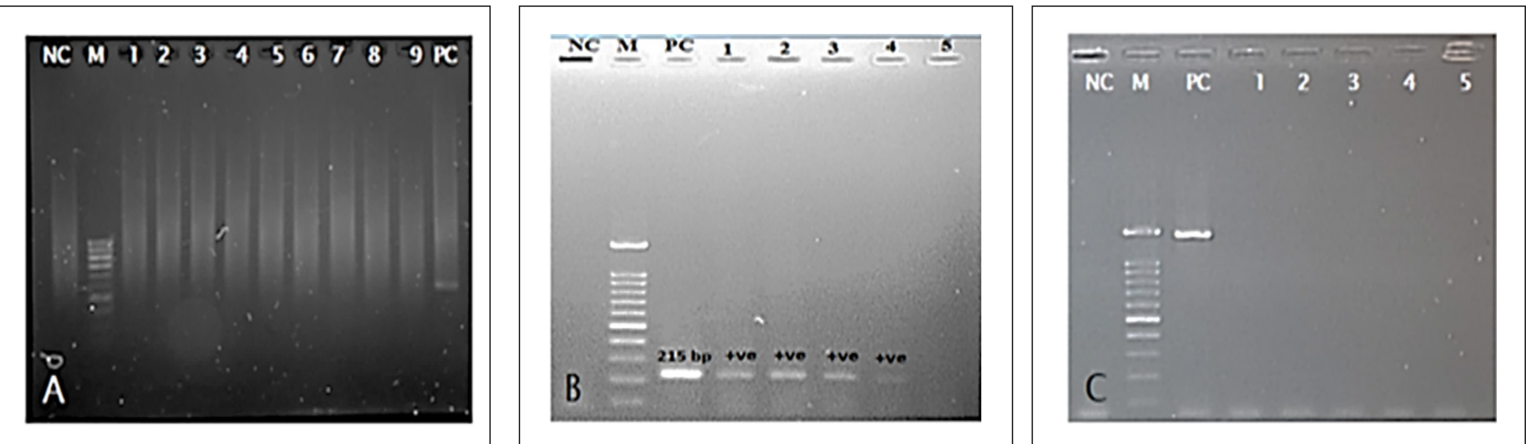


*Figure 1*. Gel electrophoresis analysis of WNV (A), herpesvirus (B), and parvovirus (C) nucleic acid. No bands are observed for all samples (A, C). Samples 1 to 4 showed the presence of bands at 215 bp, which is absent in sample 5 (B)
*Note*. NC = Negative control; M = Ladder; PC = Positive control

| Description | Scientific Name | Max Score | Total Score | Query Cover | E value | Per. Ident | Acc. Len | Accession |
|---|---|---|---|---|---|---|---|---|
| Acomys herpesvirus SVMS 226,222 DNA polymerase gene, partial cds | Acomys herpesvirus SVMS 226,222 | 56.5 | 56.5 | 16% | 0.001 | 100.00% | 231 | HQ587046.1 |
| Turtle herpesvirus isolate 6217 DNA-directed DNA polymerase gene, partial cds | Turtle herpesvirus | 54.7 | 54.7 | 16% | 0.004 | 100.00% | 181 | KX374559.1 |
| Gorilla beringei beringei lymphocryptovirus 1 isolate V-240 DNA polymerase (DPOL) gene,... | Gorilla beringei beringei lymphocrypto... | 54.7 | 54.7 | 16% | 0.004 | 100.00% | 229 | KU736788.1 |
| Gorilla beringei beringei lymphocryptovirus 1 isolate B-26 DNA polymerase (DPOL) gene,... | Gorilla beringei beringei lymphocrypto... | 54.7 | 54.7 | 16% | 0.004 | 100.00% | 229 | KU736786.1 |
| Columbid alphaherpesvirus 1 isolate 903/19 DNA-dependent DNA polymerase gene, parti... | Columbid alphaherpesvirus 1 | 54.7 | 54.7 | 16% | 0.004 | 100.00% | 234 | MW625939.1 |
| Hipposideros diadema herpesvirus gB, DPOL genes for glycoprotein B, DNA polymerase,... | Hipposideros diadema herpesvirus | 54.7 | 54.7 | 16% | 0.004 | 100.00% | 3741 | AB490083.2 |
| Panulirus argus virus 1 DNA-directed DNA polymerase (pol) gene, partial cds | Panulirus argus virus 1 | 54.7 | 54.7 | 16% | 0.004 | 100.00% | 177 | DQ465025.1 |

*Figure 2*. The BLAST analysis of herpesvirus DNA from one positive isolate. It shows 100% similarity to the subfamily *Betaherpesvirinae*

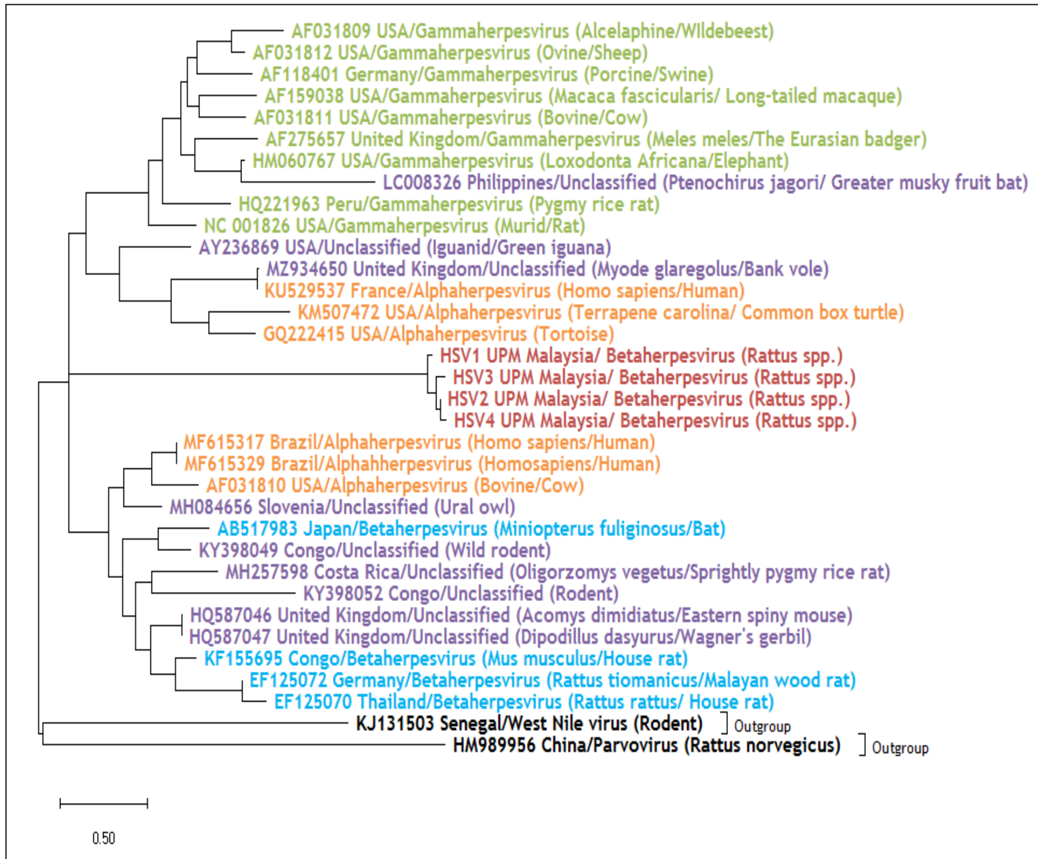


*Figure 3.* Phylogenetic analysis of herpesvirus in *Rattus* spp. (red) with 28 reference strains of herpesviruses targeting the DNA polymerase (DPOL) gene and two outgroups (black) from WNV and parvovirus. The strains were coloured in green gamma herpesvirus (9 strains), purple for unclassified herpesvirus (9 strains), orange for alpha herpesvirus (6 strains), and blue for beta herpesvirus (4 strains) targeting DPOL gene from multi-species. The tree was constructed using the maximum likelihood method with the General Time Reversible (GTR) model with 1,000 bootstrap replicates

on the strains of herpesvirus, i.e., alpha herpesvirus (orange), beta herpesvirus (blue/red), gamma herpesvirus (green), and unclassified herpesvirus (purple), as illustrated in Figure 3.

## DISCUSSION

Understanding the risk of the virus spreading from wild rats to laboratory animals may help enhance the quality of bio-life-related research. In this study, the higher detection of herpesvirus in *Rattus* spp. suggested an increased occurrence of herpesvirus even in a small sample of the rat population. Although *R. rattus* is more predominant than *R. norvegicus*, both species were positive for herpesvirus. It is similar to the finding that nearly 50% of the rat populations in Panama were positive for herpesvirus DNA (Rabson et al., 1969). The widespread of herpesvirus implies that it is ubiquitous in *Rattus* spp. because once the rats become

infected, the virus persists for a lifetime in the host before the latent infection is established. The latency could be reactivated when the host is immunocompromised, leading to asymptomatic virus shedding or manifestation of clinical signs. The close contact transmission further provides an opportunity for the virus to be largely spread in the confined area of the rat population (Barthold et al., 2016).

Identifying the *Betaherpesvirinae* sequence that signifies the RCMV indicates that herpesvirus reflects species specificity and adaptation to their host for a long time. Although beta and gamma herpesviruses are usually considered hosts specific to the herpesvirus subfamilies, they can cross species barriers and establish endemic infections (Azab et al., 2018). Novel herpesvirus may exist in the *Rattus* spp. considering the diversity of rodent species. It could become an issue as gamma herpesviruses contribute to zoonotic infections that trigger public health concerns. It is possible because gamma herpesviruses were detected previously in other rodent species. Therefore, *Rattus* spp. is a potential host for many unknown herpesviruses and has become a threat to urban communities as a reservoir for human diseases (Ntumvi et al., 2018).

The negative results of WNV and parvovirus RT-PCR from this study could be due to the non-viraemic state of the infection, i.e., the absence of the virus in the blood during the collection of blood samples. The negative results of all samples for WNV RNA indicate the absence or low transient viremia load in *Rattus* spp. (Hirota & Shimizu, 2013). Besides, the blood serum sample used in this experiment is suitable enough to detect WNV RNA with sufficient viraemic levels. However, in a low viraemic load, WNV may persist at the site of viral shedding, especially in faeces, urine, and oropharyngeal swabs (Root et al., 2005). Meanwhile, parvovirus might be found in blood early in the disease, where viremia can persist for 2 to 10 days after infection (Kilham, 1966), rendering the infected rats to be detected afterwards. The virus is most frequently detected in spleens and mesenteric lymph nodes (Wan et al., 2006), potentially increasing the chances of virus detection than the blood sample. Although the rats may be infected with parvovirus, low viral loads implicate the negative results as the PCR assay could not detect the virus. The assay is influenced by the successful recovery of live viruses from the blood samples. However, non-viable viruses or undetectable viral loads might render unsuccessful PCR assay. Both viruses were undetectable due to the low prevalence of the pathogen in the population sampled or no exposure to the virus infection.

The small sample size decreases the chances of detecting virus infection within a population. Although negative results were obtained, these results are still in agreement with other studies. A previous study showed a 2.0% and 3.3% prevalence of parvovirus in 63,808 rats in North America and Europe (Pritchett-Corning et al., 2009). In another study, 10.4% of 162 rats were serologically positive for the KRV and Toolan's H-1

virus (Easterbrook et al., 2008). The studies demonstrated the low prevalence of rat parvovirus, similar to the current study. In addition, the low molecular prevalence could be due to the clearance of viruses by antibody responses. The antibody response limits virus replication as a critical barrier to combat infection (Murin et al., 2019). A serological assay should also be conducted to conclude whether the rats are free from these three viruses.

Nevertheless, the *Rattus* spp. within Klang Valley still carry a potential risk of causing serious health implications to the public besides biosecurity risk towards laboratory rodent colonies. The incidence of rat-associated zoonoses may rise due to the proximity of rats and communities in urban neighbourhoods. Due to impoverished urban populations, environmental factors may influence zoonotic diseases associated with rats, including poor sanitation, crowding, and homelessness. The availability of food sources in food premises may also influence the abundance of rats due to improper food storage and organic waste disposal. Therefore, the rat population still need to be controlled as they can harbour diseases that raise public health concerns.

## CONCLUSION

Four rats were infected with herpesvirus, with 100% similarity to rat cytomegalovirus. Although the results suggest the circulation of herpesvirus in *Rattus* spp. in Klang Valley, they were less likely to be infected by WNV and parvovirus.

## CONFLICT OF INTERESTS

The authors declare no conflict of interest regarding the publication of this paper.

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# The Potential of Liquid Waste from the Fruit Preserves Production Process as a Low-cost Raw Material for the Production of Bacterial Cellulose

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## ABSTRACT

The liquid waste from the production of fruit preserves was used as an alternative carbon source to replace sugar in the traditional Hestrin-Schramm (HS) and coconut water media (CM) and reduce the cost of bacterial cellulose (BC) production. The sugar components of liquid wastes from preserved tamarind (LWT) and preserved mango (LWM) were characterized, and the total sugars were between 237.50 g/L and 231.90 g/L. The effects of the nutrients in the media with LWT and LWM on the production of BC by *Acetobacter xylinum* were determined. The result showed that *A. xylinum* could grow and produce BC in the media with liquid waste. The highest concentration of BC, 6.60±0.04 g/L, was obtained from the medium containing 25% (v/v) LWM. In a medium containing LWT, *A. xylinum* produced a maximum BC of 5.50±0.30 g/L when 12.5% (v/v) LWM was added. However, when the structure and physical properties of the BC from the liquid waste were characterized, it was similar to BC from the HS medium and CM medium without liquid waste.

*Keywords*: Bacterial cellulose, fruit preserve process, liquid waste, low-cost carbon source, mango, tamarind

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## INTRODUCTION

Bacterial cellulose (BC) is the fiber produced by microbes, particularly by acetic acid bacteria, such as *Acetobacter xylinum* (Aswini et al., 2020; Kongruang, 2008) and the fiber component is the bound cellulose

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structure. However, BC differs significantly from plant cellulose because BC does not contain lignin or hemicellulose (Klemm et al., 2001; Rahman & Netravali, 2016). In the past, BC was produced using raw food materials with additional ingredients, resulting in cellulose with better mechanical properties, such as high-water storage capacity or expansion ability. The best-performing BC can be used to develop many products, such as packaging, artificial skin, film, and stabilizer (Cacicedo et al., 2016; Esa et al., 2014; Ruka et al., 2014). Currently, the production of BC needs to be augmented.

In general, BC fiber may be produced by the cultivation of bacteria through a fermentation process. Coconut water has been used as a carbon source for microbial growth to produce cellulose fibers. Currently, coconut water is of greater value. It has higher market demand because it is a food and can be applied to cosmetic products with high-value BC because the competition for raw materials is increasing. However, the current price of coconuts is increasing due to increased demand, which affects the cost of BC produced from coconut water. Many reports have found that BC can be produced from many raw agricultural materials (Akintunde et al., 2022; Azeredo et al., 2019), such as pineapple juice, coconut milk, and skim milk, can produce a BC product, but the odor is significantly down to the raw material to the product. Consequently, the raw materials that produce BC are diverse and can be found local to production (Costa et al., 2017; Thongwai et al., 2022), so it is important to consider the nutrients and the appropriate state for microbial development.

This study focused on liquid waste from the fruit preservation process. The fruit preservation process is a sweet fermentation (called “Chair Im”). After fermentation, most of the ingredients in the fruit are sugars; the liquid waste contains lactic acid (Olszewska-Widdrat et al., 2020). If waste contaminates rivers or reservoirs, it will result in an algal boom or eutrophication because liquid waste is a source of energy for environmental microorganisms (Vollstedt et al., 2020). The purpose of this study was to evaluate the liquid waste from the fruit preservation process in the production of BC. The result of the study is the feasibility of applying this waste or other local waste products to the continuous production of BC.

## MATERIALS AND METHODS

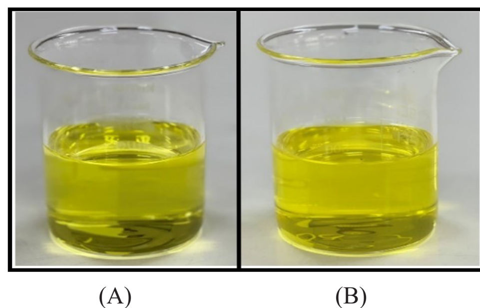
### Preparation of Inoculum

A bacterial strain of *Acetobacter xylinum* was obtained from the Thailand Institute of Scientific and Technological Research (TISTR), Thailand. *Acetobacter xylinum* was transferred to Hestrin-Schramm medium (HS) (Hestrin & Schramm, 1954; Nguyen et al., 2022), which contained 20 g of glucose (SCHARLAU, Spain), 5 g of peptone (SCHARLAU, Spain), 5 g of yeast extract (SCHARLAU, Spain), 2.7 g of disodium hydrogen phosphate ($Na_2HPO_4$) (QRëC™, New Zealand), and 1.5 g of citric acid (SCHARLAU, Spain) per 1 liter (L) of media. The pH of the medium was adjusted

to 4.2 and incubated at 30 °C for 3 days. After that, 10% (v/v) of the culture was transferred to a fresh HS medium within 48 hours of incubation. Then, the culture was used as the initial inoculum for BC production.

## Production of BC

The liquid waste from the fruit preservation process consisted of waste from tamarind preserves (LWT) and mango preserves (LWM) was used throughout this study, presented in Figure 1. The raw material was collected from the Talad Thai market area of Pathum Thani Province, Thailand. The liquid waste from the fruit preserve process was first filtered through a cotton sheet, and some physicochemical properties of the liquid waste were characterized. Then, high-performance liquid chromatography (HPLC) used the supernatant for sugar content analysis. The total soluble solid content was measured using a refractometer, while the pH was measured with a pH meter. Then, the sample was kept at 20 °C to prevent microbial contamination. It was left at room temperature and sterilized before use.



(A) (B)

*Figure 1*. Liquid waste from mango (A) and tamarind (B) preservation processes

The HS medium contains free sucrose: the addition of solid fruit waste compensates for the sucrose component in the experimental medium. The BC production of the medium utilizing liquid waste as a carbon source is compared with the BC production of the HS medium and the coconut medium (CM). The components and concentrations of the media containing LWT and LWM are presented in Table 1. After preparation, the medium was sterilized in an autoclave at 121 °C for 15 min, 10% (v/v) inoculum was added, covered with Whatman No. 1 filter paper, and incubated at 30 °C for 10 days to achieve static fermentation.

## The Characterization of BC

After fermentation, the BC film was washed and soaked thoroughly in distilled water for 2–3 days. Then, it was boiled in 1% (w/v) sodium hydroxide solution (NaOH) (SCHARLAU, Spain) for 1 h and washed with distilled water until the pH measured about 7.0. Next, the wet weight of the BC film was determined. After that, the sample was dried at 60 °C until it reached a constant dry weight, and the moisture content of BC was calculated to investigate water loss after drying. Finally, the structure of the dried BC film has been characterized using Fourier transform infrared (FTIR) spectroscopic scanning between 4,000 and 400 $cm^{-1}$ to explain a structure-function group of BC (Gea et al., 2011).

Table 1

*Components and concentrations of media with LWT and LWM*

| Components | HS | CM | LWT | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 01 | 02 | 03 | 04 | 05 | 06 | 07 | 08 | 09 | 10 | 11 | 12 | 13 |
| Glucose (g/L) | 20 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Peptone (g/L) | 5 | 5 | 5 | 5 | – | – | 5 | 5 | – | 5 | – | 5 | – | – | – |
| Yeast (g/L) | 5 | 5 | 5 | – | 5 | – | 5 | 5 | 5 | – | 5 | – | – | – | – |
| $Na_2HPO_4$ (g/L) | 2.7 | 2.7 | 2.7 | 2.7 | 2.7 | – | – | 2.7 | – | – | 2.7 | 2.7 | 2.7 | 2.7 | – |
| Citric acid (g/L) | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | – | 1.5 | – | 1.5 | 1.5 | – | – | 1.5 | – | 1.5 |
| LWT (%v/v) | – | – | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 |
| Coconut water (L) | – | 1 | – | – | – | – | – | – | – | – | – | – | – | – | – |
| $H_2O$ (L) | 1 | – | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | LWM | | | | | | | | | | | | | | |
| | HS | CM | 01 | 02 | 03 | 04 | 05 | 06 | 07 | 08 | 09 | 10 | 11 | 12 | 13 |
| Glucose (g/L) | 20 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Peptone (g/L) | 5 | 5 | 5 | 5 | – | – | 5 | 5 | – | 5 | – | 5 | – | – | – |
| Yeast (g/L) | 5 | 5 | 5 | – | 5 | – | 5 | 5 | 5 | – | 5 | – | – | – | – |
| $Na_2HPO_4$ (g/L) | 2.7 | 2.7 | 2.7 | 2.7 | 2.7 | – | – | 2.7 | – | – | 2.7 | 2.7 | 2.7 | 2.7 | – |
| Citric acid (g/L) | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | – | 1.5 | – | 1.5 | 1.5 | – | – | 1.5 | – | 1.5 |
| LWM (%v/v) | – | – | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 |
| Coconut water (L) | – | 1 | – | – | – | – | – | – | – | – | – | – | – | – | – |
| $H_2O$ (L) | 1 | – | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

*Note*. LWT = Liquid waste from tamarind preserve; LWM = Liquid waste from mango preserve; HS = Hestrin-Schramm medium; CM = Coconut medium

## RESULTS AND DISCUSSION

### Physicochemical Composition of Liquid Waste from the Fruit Preservation Process

Sour tamarind and sour mango are the most preserved fruits because of their sour taste, and this is a seasonal fruit that will have a great annual seasonal impact due to overconsumption. However, the tamarind and mango preservation process use as much as 70% sugar, so this study uses liquid waste from tamarinds and mangos as the raw material to produce BC. The LWT has a pH of 2.66, which is lower than the pH of LWM is 2.72. The total soluble solids of LWT contained 26.3 °Bx of total sugar (237.50 g/L), and the total soluble solids of LWM had 24.6 °Bx of total sugar (231.90 g/L). The sugar components of the liquid waste were in the order sucrose > glucose > fructose by HPLC analysis (Table 2). The ratio of sucrose, glucose, and fructose in the LWT was 20:1:1; the ratio was similar for LWM. However, the fermentation used to preserve food uses sugar, resulting in liquid waste with a high sugar concentration (Amit et al., 2017; Zahan et al., 2017).

Table 2

*Composition of LWT and LWM*

| | Composition | LWT | LWM |
|---|---|---|---|
| Sugar (g/L) | Total sugar | 237.50 | 231.90 |
| | Fructose | 10.40 | 11.40 |
| | Glucose | 10.50 | 10.50 |
| | Sucrose | 216.60 | 210.00 |
| pH | | 2.66 | 2.72 |
| Total soluble solid (°Brix [a]) | | 26.3 | 24.6 |
| Nitrogen | | 0 | 0 |

*Note.* [a]Degrees Brix (°Bx) is the sugar content of an aqueous solution; One degree Brix is 1 g of sucrose in 100 g of solution. LWT = Liquid waste from tamarind preserves; LWM = Liquid waste from mango preserves

LWT and LWM were potential carbon and energy sources for culturing *A. xylinum* for BC production. The HS medium used for BC production generally contains not less than 20 g/L of sugar. The liquid waste has high sugar: 8.5% (v/v) liquid waste was added to make a sugar concentration of 20 g/L in the medium. However, the minimum amount of glucose to support BC culturing was 5 g/L (Çakar et al., 2014; Chen et al., 2019; Nguyen et al., 2022).

**Production of BC from Liquid Waste from the Fruit Preservation Process**

The *A. xylinum* growth and BC production in LWT and LWM were better than in the HS and CM, and the *A. xylinum* produced a high yield of BC from LWT01 and LWM01, which produced an average dry weight of BC of 4.76±0.16 and 4.43±0.91 g/L, respectively. The organic and inorganic nitrogen and citric acid were added to the media, and the yield of BC dry weights is presented in Figure 2.

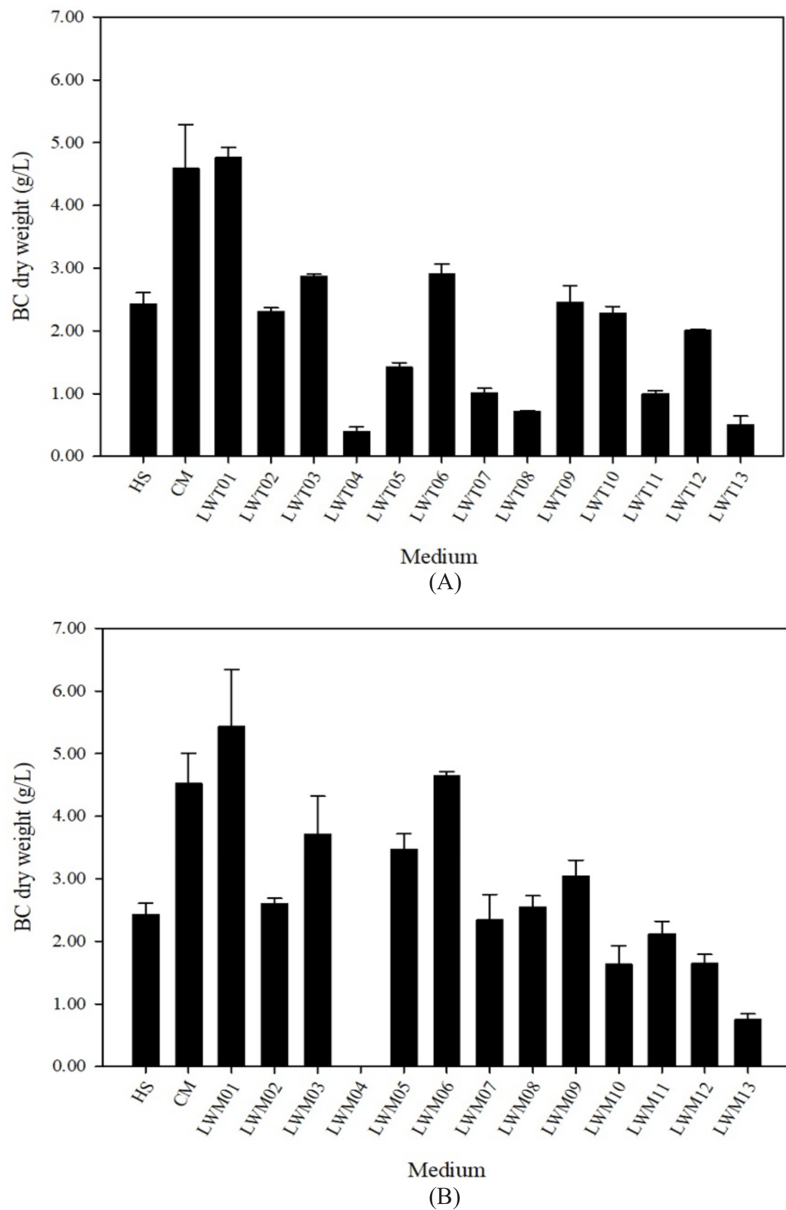



*Figure 2*. BC produced by *A. xylinum* in (A) LWT and (B) LWM (B), respectively

Then the LWT and LWM volume was adjusted to 8.5%, 12.5%, 17%, 25%, and 35% (v/v), with corresponding total sugar concentrations of 20, 30, 40, 60, and 80 g/L. Other components were added to the HS medium formulation before sterilization in the autoclave, inoculation with 10% (v/v) *A. xylinum*, and incubation at 30 °C for 10 days.

The BC production had a high yield of 6.60±0.04 g/L of dry weight in LWM 25%, and the carbon sources from LWT had potential BC products of 5.50±0.30 g/L when 30 g/L (12.5%) sugar was used in the medium. However, in the medium, BC cannot be produced at sugar ratios of 25% and 35% or sugar concentrations of 60 g/L and 80 g/L.

Increasing the sugar concentration of the medium from 20 g/L or adding more than 8.5% liquid waste to the LWT and LWM media increased the BC. However, in LWM, the fiber of bacteria decreased when the sugar concentration was increased to 80 g/L, or 35% liquid waste was added. The BC cannot be produced in the media containing LWT with sugar concentrations of 60 g/L (25%) and 80 g/L (35%). Perhaps the elevated sugar concentration affects growth because of the inhibition of the substrate (Dikshit & Kim, 2020; Kouda et al., 1998; Väljamäe et al., 2001). The yield of BC is presented in Figure 3.

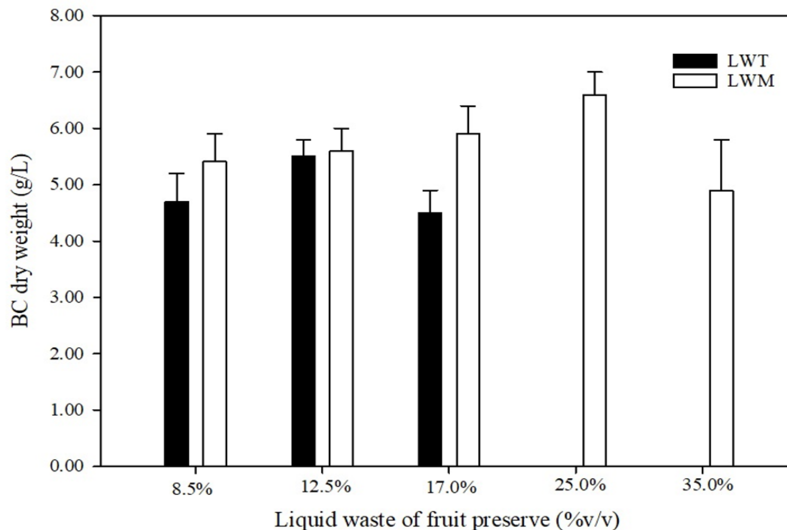



*Figure 3*. BC produced by *A. xylinum* and bacteria strain in HS medium liquid waste of fruit preserve between LWT and LWM

The pH of LWT and LWM decreased after the BC was harvested (Table 3). Photographs of the washed BC produced are presented in Figure 4(A), and the dry BC is presented in Figure 4(B). The moisture content of BC ranged between 96–97%; this result means that the fiber structure of BC can hold large amounts of water (Rebelo et al., 2018; Schrecker & Gostomski, 2005).

Table 3

*The volume of cellulose produced and properties of fiber from LWM and LWT*

| Liquid waste | Volume (% v/v) | Total concentration in liquid waste (g/L) | pH | | % Moisture content |
|---|---|---|---|---|---|
| | | | Initial | Final | |
| LWM | 8.5 | 20.0 | 4.2 | 3.94 | 96.68 |
| | 12.5 | 30.0 | 4.2 | 3.97 | 97.24 |
| | 17.0 | 40.0 | 4.2 | 3.91 | 96.68 |
| | 25.0 | 60.0 | 4.2 | 3.87 | 97.21 |
| | 35.0 | 80.0 | 4.2 | 3.71 | 97.17 |
| LWT | 8.5 | 20.0 | 4.2 | 3.85 | 96.64 |
| | 12.5 | 30.0 | 4.2 | 3.97 | 97.23 |
| | 17.0 | 40.0 | 4.2 | 3.91 | 96.52 |
| | 25.0 | 60.0 | 4.2 | 3.72 | 0.00 |
| | 35.0 | 80.0 | 4.2 | 3.63 | 0.00 |

*Note*. LWT = Liquid waste from tamarind preserves; LWM = Liquid waste from mango preserves

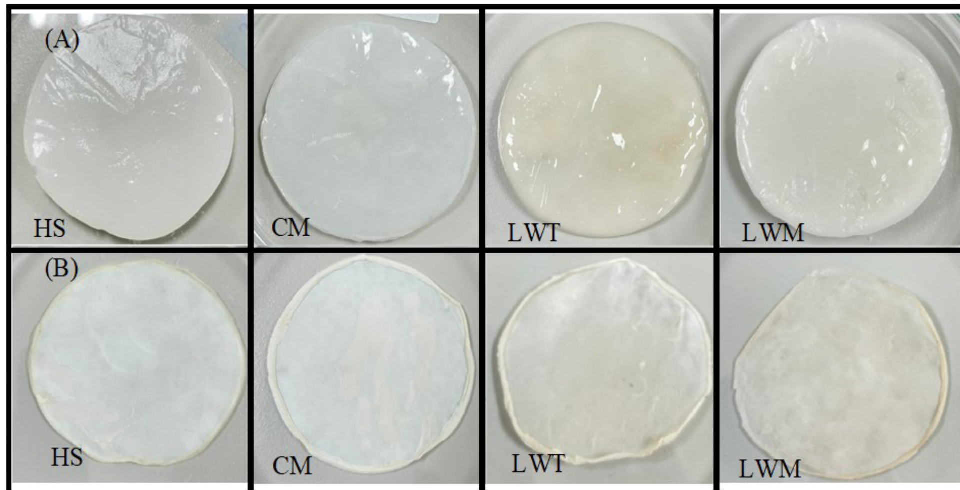



*Figure 4*. (A) Wet and (B) dry BC produced from *A. xylinum* in different media

The FTIR spectra of BC produced from LWT and LWM were similar to those of BC from HS and CM, presented in Figure 5. The peak between 665–670 cm$^{-1}$ is assigned to the out-of-plane with a carbon-hydrogen bond (C-H), bending the carbon bond of carbon to the hydroxyl group (δ(C-OH)). The peak at about 1,111 cm$^{-1}$ is assigned to the alkanes stretch (ʊ(C-C)) ring group of polysaccharides and cellulose (Kacuráková et al., 2002; Movasaghi et al., 2008), and the peaks at 900 cm$^{-1}$ and about 1249 cm$^{-1}$

are assigned to the carbonyl group (C=O) and ether (C−O−C) stretching of glucose (Carrillo et al., 2004; Wong et al., 2009). The peaks at 3,278 and 3,345 $cm^{-1}$ are assigned to stretching oxygen-hydrogen bond (Ս(O-H)) of cellulose I (Moharram & Mahmoud, 2007).

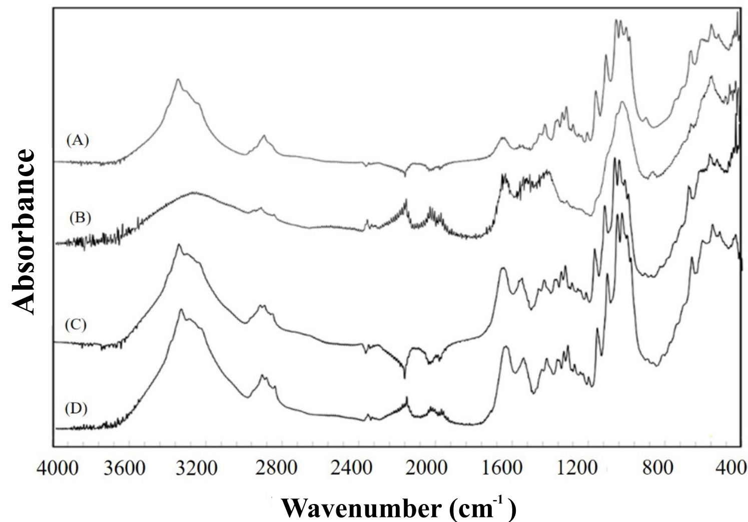



*Figure 5*. FTIR spectra of BC films produced from *A. xylinum* in (A) HS medium, (B) CM, (C) LWT, and (D) LWM

## CONCLUSION

Liquid waste from the fruit preservation process can be used as the raw material for *A. xylinum* growth and the production of BC because it contains mainly sugar compounds. Moreover, the BC produced from this liquid waste has a structure similar to that of the BC produced from coconut water. Therefore, liquid waste has the potential as an alternative carbon source in BC production to reduce costs. The sugar ratio of LWT and LWM is similar, sucrose > glucose > fructose (20:1:1), and the sugar concentration in the liquid waste can be mixed in the medium for BC production. The results of this study show that *A. xylinum* grew and produced BC in LWT and LWM higher than in HS and CM. The BC production had the highest yield in LWM, 25%. The BC cannot be produced in a medium containing LWT with a sugar concentration over 60 g/L (25%), possibly because the high sugar concentration affects the growth due to substrate inhibition. However, the moisture content of the resulting BC ranges between 96–97%; the fiber structure of this BC can hold a large amount of water. This study is initial research for using waste from agricultural production to add value and decrease waste. In the future, it is important to continue developing uses for BC in the industrial sector.

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# Toxicity of Malaysian Medicinal Plant Extracts Against *Sitophilus oryzae* and *Rhyzopertha dominica*

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## ABSTRACT

The insecticidal activities of extracts from 22 Malaysian medicinal plant extracts from 8 botanical families were tested against rice weevil: *Sitophilus oryzae* (L.) and lesser grain borer: *Rhyzopertha dominica* (F.). The extracts were obtained using hexane, methanol, and dichloromethane to extract potential biopesticides from dried leaves. The toxicity levels were examined periodically based on antifeedant activity and contact toxicity assays using treated grain assay. Hexane extracts of *Alpinia conchigera*, *Alpinia scabra*, *Curcuma mangga*, *Curcuma purpurascens*, *Goniothalamus tapisoides*, *Piper sarmentosum*, and methanol extracts of *Curcuma aeruginosa*, *C. mangga*, and *Mitragyna speciosa* were the most potent extracts against *S. oryzae and R. dominica*

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with lethal concentration ($LC_{50}$) values of ≤ 0.42 mg/mL and ≤ 0.49 mg/mL, respectively. The contact toxicity test results showed that methanol extracts of *C. aeruginosa* and *C. mangga*, dichloromethane extracts of *Cryptocarya nigra*, and hexane extracts of *C. mangga*, and *C. purpurascens* resulted in 100% mortality of both pests within 28 days exposure of 5 mg/cm$^2$ concentration.

*Keywords*: Antifeedant, insecticidal activity, lesser grain borer, Malaysian medicinal plant, rice weevil

## INTRODUCTION

The quality of stored grains and related food products can be affected severely due to insect and pest invasions. Even with proper measures, the damage caused to stored grains and food products by insects can be as high as 10%, reaching 30% in tropical countries (Nakakita, 1998, as cited in Rajashekar & Shivanandappa, 2010, pg. 910). Insect pests were partly responsible for losses of grains (wheat, maize, rice, and soybean) in pre-harvest, post-harvest, and storage, which total up to 1.741 mt (Mesterházy et al., 2020). For example, a report revealed that up to 0.557 million mt rice losses in Malaysia from the post-harvest losses (PHL) activities (Hamzah, 2017, as cited in Nodin et al., 2021, pg. 43). Since their introduction in the 1940s, synthetic insecticides are widely used to protect grains from insect infestation (MacIntyre, 1897). Some commonly used insecticides to control storage pests include organophosphates (e.g., diazinon and chlorpyrifos) and pyrethroids (e.g., deltamethrin and resmethrin). In addition, malathion and pirimiphos-methyl are often present as residual insecticides in stored products (Andrić & Pražić-Golić, 2014; Obeng-Ofori, 2010; World Health Organization [WHO], 1997). However, the lack of proper measures and indiscriminate use of synthetic insecticides has led to the widespread development of resistance, environmental pollution, and negative impact on natural enemies and health hazards to humans (Akhtar et al., 2007; Gill & Garg, 2014; Subramanyam & Hagstrum, 2020). For example, *S. oryzae* (L.) and *R. dominica* (F.), which are considered as main pests of stored food (Majeed et al., 2015), have developed levels of resistance to organophosphorus class pesticides, leaving with few options for their control (Sadeghi & Ebadollahi, 2015; Talukder, 2009). Therefore, it is a dire need to develop safer alternatives as well as insecticides with a target-selective and novel mode of action to reduce the adverse effects on non-targeted insects and natural enemies of insect pests.

Plants have long been considered a potential source of bioactive phytochemicals that can be exploited for the discovery of newer agents for insect control (Adeniyi et al., 2010; Wink, 1993). Through many studies, plant-derived secondary metabolites have been proven suitable for integrated pest management (IPM) use. Based on an IPM ecosystem-oriented strategy, the main target is the long-term prevention of the specific insects' pests, preferably by using a botanical pesticide. The superiority of biopesticides over commonly available ones are due to their several advantages, including biodegradability, selective toxicity to targeted insects, and less toxic

to mammals, suggesting the need for developing a new class of biopesticides for insect control (Joseph et al., 2012; Salunke et al., 2009). Despite worldwide efforts to find alternatives for synthetic insecticides, there is still no botanical insecticide developed that is potent enough to replace synthetic insecticides to protect stored grains. With a rich floral diversity of about 15,000 species, Malaysian plants represent a potential source for the exploration of botanical insecticides (Ministry of Natural Resources and Environment [KeTSA], 2006; Sivapragasam, 2009). Mousa et al. (2011) showed that extracts of botanical plants, *Piper nigrum* and *Jatropha curcas*, contained compounds of piperine, oleic acid, linoleic acid, caryophyllene, and palmitic acid, were able to protect stored rice against *S. oryzae*.

Therefore, this study investigated the potential insecticidal activities of several Malaysian plants used in traditional medicine or bioactive properties. Insecticidal activity of hexane, dichloromethane, and methanol extracts of 22 Malaysian plants from the families Annonaceae, Apocynaceae, Calophyllaceae, Lauraceae, Meliaceae, Piperaceae, Rubiaceae, and Zingiberaceae were assessed by a range of bioassays against adult rice weevil (*Sitophilus oryzae*) (Coleoptera: Curculionidae) and lesser grain borer (*Rhyzopertha dominica*) (Coleoptera: Bostrychidae).

## MATERIALS AND METHODS

### Insects

Mixed-sex adults of *S. oryzae* and *R. dominica* (1- to 2-week-old) were used in this study. They were kept in the laboratory at a temperature and relative humidity of 26 ± 1ºC and 65 ± 5%, respectively, for 12 hours daylight and 12 hours dark and fed with hard rice supplemented with 5% yeast (Saf-instant, France). These insects have been reared in the laboratory for two generations.

### Plant Materials and Extraction

More than 20 Malaysian plant species with medicinal properties or traditional uses were selected for extraction (Table 1). Botanical identification of the collected plant samples was performed by Prof. Dr. Halijah Ibrahim and Mr. Teo Leong Eng from the Faculty of Science, Universiti Malaya, and Prof. Dr. Kamarudin Mat Salleh from the Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The voucher specimens were deposited at the Herbarium of the Department of Chemistry, Universiti Malaya.

All the dried plant materials (1 kg) were macerated with hexane (17 L) (R&M Chemical, Malaysia) for 3 days. Then, the hexane extracts were evaporated to dryness under reduced pressure using a rotary evaporator. The hexane extraction process was repeated twice to maximize the extraction yield. First, the same extraction process was repeated by using dichloromethane (DCM) (R&M Chemical, Malaysia) (ethyl acetate for *Mitragyna speciose*, *Mesua elegans*, and *Mesua kunstleri*) followed by methanol (MeOH) (R&M Chemical, Malaysia).

Table 1
*Selected plants for insecticidal activity investigation with their traditional uses, biological/pharmacological properties, types of chemical constituents, and major compounds contained in the plants*

| Plant families/species | Voucher specimen | Part used | Traditional uses/ Biological/ Pharmacological properties | Types of chemical constituents | Major compounds | References |
|---|---|---|---|---|---|---|
| **Annonaceae** | | | | | | |
| *Goniothalamus tapisoides* | HUMS 000108 | Bark | - Used as abortifacient and to cure poisonous animal bites, such as snake, scorpion sting, or insect bites, and to relieve stomachache | Styryl lactones, alkaloids, and flavonoids | Goniothalamin | Ahmad et al. (2010); Kim et al. (2012) |
| **Apocynaceae** | | | | | | |
| *Alstonia angustifolia* | KL 5705 | Leaves | - Used for treatment of remittent fever by applying to the spleen area | Indole alkaloids | Alstogustine and 19-epialstogustine | Naeem et al. (2017); Perry and Metzger (1980); Raja et al. (2013); S. Aziz et al. (2016) |
| *Catharanthus roseus* | KL 5763 | Leaves | - Used for treatment of wasp stings, menorrhagia, and rheumatism<br>- Anti-cancer, antioxidant, and antidiabetic activities | Indole alkaloids | Vindoline I, vindolidine II, vindolicine III, and vindolinine IV | Tiong et al. (2013) |
| **Calophyllaceae** | | | | | | |
| *Mesua elegans* | KL 5232 | Bark | - Acetylcholinesterase inhibitory activity | Coumarins | Mesuagenin A and Mesuagenin D | Awang, Chan, et al. (2010b) |
| *Mesua kunstleri* | KL 4485 | Bark | - Neuroprotective activity | Coumarins | 5,7-dihydroxy-6-(3-methylbutanoyl)-8-[*E*-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one | Chan et al. (2012) |

Table 1 *(Continue)*

| Plant families/ species | Voucher specimen | Part used | Traditional uses/ Biological/ Pharmacological properties | Types of chemical constituents | Major compounds | References |
|---|---|---|---|---|---|---|
| **Lauraceae** | | | | | | |
| *Alseodaphne corneri* | KL 5641 | Bark | - Antiplasmodial and antioxidant activities | Bisbenzylisoquinoline, aporphine, and benzylisoquinoline alkaloids | Gyrolidine and isocorydine | Zahari (2016) |
| *Dehaasia longipedicellata* | KL 5634 | Bark | - Antiplasmodial and antioxidant activities | Morphinandienone, aporphine, and benzylisoquinoline alkaloids | Sebiferine and milonine | Zahari et al. (2014) |
| *Cryptocarya nigra* | KL 5272 | Bark | - Antiplasmodial and antioxidant activities | Benzylisoquinoline and phenanthrene alkaloids | *N*-methylisococlaurine | Nasrullah et al. (2013) |
| *Phoebe grandis* | KL 5532 | Leaves | - Cytotoxic and antibacterial activities | Aporphine, proaporphine, and proaporphine-tryptamine dimers | | Amna et al. (2015); Mukhtar et al. (1997) |
| **Meliaceae** | | | | | | |
| *Walsura pinnata* | KL 4571 | Bark | - Antiproliferative and anti-colonogenic properties | Triterpenes | 3-oxoolean-1-en-28-oic acid and betulonic acid | Leong et al. (2017); Mohamad et al. (2009) |
| **Piperaceae** | | | | | | |
| *Piper sarmentosum* | KU 0110 | Root | - Used for treatment of toothache, fungoid dermatitis on the feet, coughing, asthma, and pleurisy<br>- Larvicidal, cytotoxicity, anti-amoebic, antiplasmodial, antimycobacterial, and antifungal activities | Phenylpropanoids and pyrrole amide | Asaricin, isoasarone, and *trans*-asarone | Hematpoor et al. (2016, 2018); Sawangjaroen et al. (2004); Tuntiwachwuttikul et al. (2006) |

Table 1 *(Continue)*

| Plant families/ species | Voucher specimen | Part used | Traditional uses/ Biological/ Pharmacological properties | Types of chemical constituents | Major compounds | References |
|---|---|---|---|---|---|---|
| **Rubiaceae** | | | | | | |
| *Nauclea officinalis* | KL 5655 | Bark | - Vasorelaxant and cholinesterase inhibitory activities | Indole alkaloids and quinoline alkaloid glycoside | Angustine and naucletine | Liew et al. (2012, 2015) |
| *Nauclea subdita* | KL 5254 | Bark | - Cytotoxic activity | Indole alkaloids | Angustine and angustoline | Liew et al. (2014) |
| *Mitragyna speciosa* | KL 5321 | Leaves | - Used as an antidepressant, cure for fever, treatment for diarrhea, and diabetes, improve blood circulation, and as a morphine substitute<br>- Anti-inflammatory activity | Indole alkaloids | Mitragynine, speciociliatine, 7-hydroxymitragynine, palmitic acid, sitosterol, stigmasterol, campesterol, and tocopherol | Burkill (1935); Idayu et al. (2011); Tohar et al. (2019) |
| **Zingiberaceae** | | | | | | |
| *Alpinia conchigera* | KL 5049 | Rhizomes | - Used as a condiment and post-partum medicine<br>- Cytotoxic and antimicrobial activities | Phenylpropanoid and monoterpenes | Acetoxychavicol acetate | A. N. Aziz et al. (2013); Awang, Azmi, et al. (2010a); Ibrahim et al. (2009); Sok et al. (2017) |
| *Alpinia murdochii* | HI 1420 | Rhizomes | - Used for antimicrobial and anti-inflammatory actions | Glycoside, limonoids, and terpenoids | γ-selinene | Burkill (1935); Mohamad (2009) |
| *Alpinia pahangensis* | KU 001 | Rhizomes | - Used to relieve flatulence | Monoterpenes and sesquiterpenes (Essential oil) | (E)-labda-8(17),12-diene-15,16-dial | Awang et al. (2011); Phang et al. (2013); Sivasothy et al. (2013) |
| *Alpinia scabra* | HI 1419 | Rhizomes | - Used to cure gastric diseases and insect bites<br>- Trematocidal activity | Alkaloids, limonoids, and terpenoids | - | Burkill (1935) |

Table 1 *(Continue)*

| Plant families/ species | Voucher specimen | Part used | Traditional uses/ Biological/ Pharmacological properties | Types of chemical constituents | Major compounds | References |
|---|---|---|---|---|---|---|
| **Zingiberaceae** | | | | | | |
| *Curcuma aeruginosa* | HI 1349 | Rhizomes | - Used in postpartum care: uterine involution, treatment of uterine pain, and uterine inflammation | Alkaloids and cucumenoids | Zedoalactone A, zedoalactone B, curcumin, and β-pinene | Perry and Metzger (1980); Thaina et al. (2009) |
| *Curcuma mangga* | HI 1350 | Rhizomes | - Used in the treatment of stomachic, chest pain, fever, and general debility, postpartum, and to aid womb healing | Terpenoids, flavonoids, and cucumenoids | Zerumin B and curcumin | Abas et al. (2005) |
| *Curcuma purpurascens* | KL 5793 | Rhizomes | - Traditionally used to treat boils, cough, fever, itch, scabies, and wounds | Monoterpenes and sesquiterpenes (Essential oil) | Turmerone, germacrone, and germacrene-B | Hong et al. (2014); Koller (2009) |
| *Curcuma zedoaria* | KL 5764 | Rhizomes | - Used as an aromatic stomachic and to stimulate the blood flow | Flavonoids, cucumenoids, and sesquiterpenoids | Curzerene, dehydrocurdione, curcumenone, comosone II, curcumenol, and procurcumenol | Ahmed Hamdi et al. (2014); Lobo et al. (2009); Wilson et al. (2005) |

**Insecticidal Activity**

Previous studies showed that *S. oryzae* was more susceptible to insecticides than *R. dominica,* and thus *S. oryzae* was chosen for the preliminary test in the present study (Athanassiou et al., 2016; El-Masry, 2008). Initially, 1 mg/mL solution of each plant extract was used, and based on the observed insecticidal activity, the extracts were classified as strong (mortality ≥ 80%), moderate (mortality 50–79%), weak (mortality 30–49%), and little or no activity (mortality below 30%). Extracts with strong or moderate activity (≥ 50%) were chosen for further investigation to determine the lethal concentration, effect at various exposure intervals, contact toxicity, and antifeedant activities.

**Relative Dose Value on Treated Grain Assay**

In determining the working concentration level, 20 adults (mixed sex) of *S. oryzae* and *R. dominica* were introduced separately in Petri dishes containing 5 g rice grain treated with different active extracts. The Petri dishes were covered and sealed with parafilm. Mortality was recorded at 24, 48, and 72 hours after the exposure, and the percentage of mortality was calculated (Abbott, 1987). The untreated group was considered the control and all the experiments had four replicates.

**Effect of Exposure Interval on Pests' Survival**

Two-week-old adults of *S. oryzae* and *R. dominica* ($n$ = 20) were separately exposed for 21 days in vials containing rice grain treated with each active extract at the concentration of their $LC_{50}$ values. The mortality was recorded after exposure on the 7$^{th}$, 14$^{th}$, and 21$^{st}$ days. The experimental protocol followed the same pattern as the treated grain assay. Upon completion of the exposure time, dead insects were recorded and discarded. Results were analyzed using the analysis of variance (ANOVA) of the Statistical Analysis System (SAS) (SAS Institute, 1987) to determine the significance of exposure interval (7, 14, and 21 days) (Ferizli et al., 2011).

**Contact Toxicity**

The assessment of chronic toxicity was obtained by applying 425 mg of each plant extract dissolved in 1 mL of methanol on filter papers (Whatman No. 1, diameter 10 cm) to achieve a concentration of 5.41 mg/cm$^2$ (Kim et al., 2003). Filter paper for the control group was treated with methanol only. After drying in a fume hood for 15 minutes, each filter paper was placed at the bottom of a Petri dish (10 cm diameter x 1.5 cm), and 20 adults of *S. oryzae* and *R. dominica* were separately placed in each Petri dish. All the Petri dishes were covered and sealed with parafilm for 28 days, with checking for mortality on the 7$^{th}$, 14$^{th}$, 21$^{st}$, and 28$^{th}$ days.

**Antifeedant Assay**

The potential of antifeedant effects of the active plant extracts was studied as described by Arivoli and Tenny with slight modifications (Arivoli & Tennyson, 2013).

Briefly, 5 g of untreated organic rice was used as a test food. The rice was treated with ethanol (control group) or 5.0, 2.5, and 1.5 mg/mL solution of each extract and allowed to air-dry for 5 hours. Batches of 10 adults (2 weeks old) of *S. oryzae* and *R. dominica* were placed in each Petri dish and wrapped with parafilm. The antifeedant activity was recorded on the $28^{th}$ day after treatment (DAT). The antifeedant activity was determined using the following formula for weight loss percentage (Saad & Abdelgaleil, 2018):

$$\% \text{ weight lose} = [(Wu–Wi)/Wu] \times 100$$

where Wu = weight of uninfected rice and Wi = the final weight. Plant extracts with ≥ 70% feeding inhibition at 5.0 mg/mL were considered highly active, and further assays were carried out at lower concentrations (2.5 and 1.5 mg/mL).

### Statistical Analysis

All data were subjected to statistical analysis using ANOVA followed by Tukey's test using SAS software (version 9.1.3), and the results were considered significant when $p \leq 0.05$. The $LC_{50}$ values were calculated by probit analysis (Finney, 1971) using POLO Plus (Ahn et al., 1998; Robertson et al., 1980).

## RESULTS

### Screening for Insecticidal Activity

The preliminary test showed that the most active extracts against *S. oryzae* were from the hexane and MeOH extracts of the plant samples (Table 2). Insecticidal activity of the active plant extracts was observed when exposed to extract with a concentration of 1 mg/mL. Insecticidal activity for *Piper sarmentosum* extracts has been reported previously (Hematpoor et al., 2017). The results showed that the hexane (100%) and MeOH extracts (76.8%) exhibited strong and moderate activity after exposure to 0.5 mg/mL of extracts for 72 hrs. Based on the percentage of mortality, plants with insecticidal activity ≥ 50% after 72 hrs of exposure (18 extracts from 14 plants including hexane and MeOH extracts of *P. sarmentosum*) were selected for further studies to determine the lethal concentration, effect of time interval, antifeedant, and contact toxicity against *S. oryzae* and *R. dominica*.

### Toxicity

The toxicity of the extracts towards the adults of *S. oryzae* and *R. dominica* were investigated by evaluating their $LC_{50}$ values (concentration in mg/mL that killed 50% of the insects). The extracts were not equitoxic to both insects under investigation. MeOH extracts of *C. aeruginosa*, *C. mangga*, and *M. speciosa*, as well as hexane extracts of *A. conchigera*, *A. scabra*, *C. mangga*, *C. purpurascens*, *G. tapisoides*, and *P. sarmentosum*, were the most active extracts against both *S. oryzae* and *R. dominica* (Table 3).

Table 2

*Insecticidal activity of plant extracts 1 mg/mL against* S. oryzae *adults from treated grain assay*

| Extract | Plant[x] | Mortality (mean ± SE, %)[y,z] | | |
|---|---|---|---|---|
| | | 24 hrs | 48 hrs | 72 hrs |
| Control | - | 00.0 ± 0.0[f] | 00.0 ± 0.0[g] | 00.0 ± 00.0[d] |
| MeOH | *Alstonia angustifolia* | 4.5 ± 2.7[e] | 19.1 ± 3.4[f] | 79.1 ± 2.3[b,c] |
| | *Curcuma aeruginosa* | 13.2 ± 2.1[d] | 43.2 ± 3.1[d,e] | 67.2 ± 1.7[d] |
| | *Curcuma mangga* | 15.0 ± 2.3[c,d] | 45.2 ± 4.8[d] | 86.7 ± 2.1[b] |
| | *Dehaasia longipedicellata* | 16.4 ± 4.8[c] | 75.1 ± 3.4[b] | 100.0 ± 0.0[a] |
| | *Mitragyna speciosa* | 20.6 ± 1.2[c] | 51.6 ± 1.3[d] | 75.0 ± 3.3[c] |
| DCM | *Cryptocarya nigra* | 3.1 ± 0.4[e] | 25.1 ± 1.7[f] | 86.3 ± 3.9[b] |
| | *Curcuma purpurascens* | 10.1 ± 1.0[d] | 61.1 ± 2.0[c] | 87.2 ± 3.7[b] |
| | *Phoebe grandis* | 10.0 ± 1.5[d] | 39.5 ± 3.2[e] | 74.5 ± 2.3[c] |
| Hexane | *Alpinia conchigera* | 35.2 ± 2.3[a,b] | 75.0 ± 2.9[b] | 81.5 ± 1.1[b,c] |
| | *Alpinia scabra* | 21.8 ± 3.5[c] | 71.3 ± 5.5[b] | 100.0 ± 0.0[a] |
| | *Curcuma aeruginosa* | 7.0 ± 4.9[e] | 19.5 ± 1.3[g] | 83.3 ± 2.9[b] |
| | Curcuma mangga | 42.0 ± 1.5[a] | 94.5 ± 1.3[a] | 100.0 ± 0.0[a] |
| | *Curcuma purpurascens* | 10.1 ± 1.0[d] | 61.1 ± 2.0[c] | 88.5 ± 3.6[b] |
| | *Curcuma zedoaria* | 21.2 ± 2.1[c] | 49.2 ± 2.4[d] | 85.0 ± 2.4[b] |
| | *Goniothalamus tapisoides* | 41.1 ± 1.5[a] | 98.8 ± 0.6[a] | 100.0 ± 0.0[a] |
| | *Mesua elegans* | 13.1 ± 0.9[d] | 21.5 ± 1.3[f] | 80.0 ± 1.8[b] |

*Note*.

[x]Plant extracts showed 50% mortality or lower after 72 hrs of treatment were not included. Hexane and MeOH extracts of *P. sarmentosum* caused 100.0% and 76.8% mortality, respectively, against *S. oryzae* after exposure to 0.5 mg/mL of extracts for 72 hrs (Hematpoor et al., 2017)

[y]Each column represented the mean of four replicates, each set up with 20 adults ($n = 80$)

[z]Mean within a column followed by the same letter are not significantly different ($p \leq 0.05$)

Table 3

*Toxicity of 18 most active plant extracts against* S. oryzae *and* R. dominica

| Extract | Plant | $LC_{50}$ (mg/mL) (95 % confidence interval)[x,y,z] | |
|---|---|---|---|
| | | *Sitophilus oryzae* | *Rhyzopertha dominica* |
| MeOH | *Alstonia angustifolia* | 0.44 (0.37 to 0.50) | ≥ 0.50 |
| | *Curcuma aeruginosa* | 0.42 (0.34 to 0.50) | 0.31 (0.19 to 0.43) |
| | *Curcuma mangga* | 0.21 (0.15 to 0.26) | 0.34 (0.31 to 0.38) |
| | *Dehaasia longipedicellata* | 0.34 (0.24 to 0.43) | ≥ 0.50 |
| | *Mitragyna speciosa* | 0.30 (0.21 to 0.40) | 0.49 (0.35 to 0.65) |
| | *Piper sarmentosum* | 0.30 (0.28 to 0.35) | ≥ 0.50 |

Table 3 *(Continue)*

| Extract | Plant | $LC_{50}$ (mg/mL) (95 % confidence interval) [x,y,z] | |
|---|---|---|---|
| | | *Sitophilus oryzae* | *Rhyzopertha dominica* |
| DCM | *Cryptocarya nigra* | 0.46 (0.40 to 0.52) | ≥ 0.50 |
| | *Curcuma purpurascens* | 0.40 (0.25 to 0.55) | ≥ 0.50 |
| | *Phoebe grandis* | ≥ 0.50 | ≥ 0.50 |
| Hexane | *Alpinia conchigera* | 0.11 (0.07 to 0.15) | 0.20 (0.14 to 0.27) |
| | *Alpinia scabra* | 0.29 (0.20 to 0.38) | 0.49 (0.43 to 0.62) |
| | *Curcuma aeruginosa* | ≥ 0.50 | ≥ 0.50 |
| | *Curcuma mangga* | 0.19 (0.13 to 0.26) | 0.28 (0.21 to 0.38) |
| | *Curcuma purpurascens* | 0.33 (0.28 to 0.38) | 0.35 (0.31 to 0.40) |
| | *Curcuma zedoaria* | 0.38 (0.34 to 0.42) | ≥ 0.50 |
| | *Goniothalamus tapisoides* | 0.21 (0.11 to 0.28) | 0.35 (0.21 to 0.47) |
| | *Mesua elegans* | ≥ 0.50 | ≥ 0.50 |
| | *Piper sarmentosum* | 0.28 (0.26 to 0.31) | 0.38 (0.27 to 0.49) |

*Note*.
[x]Values were based on five concentrations, four replicates of 20 insects each
[y]Significant values determined by comparing the confidential interval of each $LC_{50}$ value
[z]$LC_{50}$ value ≥ 0.50 mg/mL was considered low insecticidal activity and was not shown

**Effect of Exposure Interval on Survival**

Plant extracts with $LC_{50}$ < 0.50 mg/mL (Table 3) were investigated further for the effect of exposure interval due to the toxicity of the extract (Table 4) by evaluating the mortality of adults of both pests upon exposure to the extracts. Results showed that the level of toxicity of the extracts increased with a longer duration of exposure. Based on the relative $LC_{50}$ values, extracts were identified with significant residual toxicity after 21 days of exposure. For MeOH extract of *P. sarmentosum*, DCM extracts of *C. nigra*, hexane extracts of *A. conchigera*, *A. scabra*, *C. mangga*, *Goniothalamus tapiosides*, and *P. sarmentosum*, the mortality of *S. oryzae* increased up to 90–100% within 21 days with *A. conchigera* and *G. tapiosides* being the most potent extracts (100% mortality). In the case of *R. dominica*, MeOH extracts of *C. aeruginosa*, *C. mangga*, *P. sarmentosum*, DCM extract of *C. nigra*, hexane extracts of *A. conchigera*, *G. tapiosides*, and *P. sarmentosum* showed the highest mortality (80–100%) within the 21-days of the experimental period. MeOH extract of *C. mangga* and hexane extract of *P. sarmentosum* were the most potent, with 100% mortality at the end of the 21-day exposure.

Table 4

*Mortality of* S. oryzae *and* R. dominica *adults exposed to rice grain treated with $LC_{50}$ concentration of each plant extract for 21 days*[*]

| Extract | Plant | Mean mortality (%) of *S. oryzae* in days after exposure | | | Mean mortality (%) of *R. dominica* in days after exposure | | |
|---|---|---|---|---|---|---|---|
| | | $7^{th}$ day | $14^{th}$ day | $21^{st}$ day | $7^{th}$ day | $14^{th}$ day | $21^{st}$ day |
| Control | No | $00.0 \pm 0.0^{d}$ | $00.0 \pm 0.0^{e}$ | $0.8 \pm 0.03^{f}$ | $00.0 \pm 0.0^{e}$ | $00.0 \pm 0.0^{f}$ | $00.0 \pm 0.0^{f}$ |
| MeOH | *Alstonia angustifolia* | $58.7 \pm 2.3^{b}$ | $59.7 \pm 4.2^{d}$ | $59.7 \pm 4.2^{d}$ | $49.2 \pm 2.5^{c}$ | $50.5 \pm 2.3^{d}$ | $50.5 \pm 2.3^{d}$ |
| | *Curcuma aeruginosa* | $53.5 \pm 2.4^{c}$ | $63.0 \pm 3.0^{c,d}$ | $66.3 \pm 4.7^{c}$ | $66.7 \pm 2.4^{a,b}$ | $83.0 \pm 2.3^{b}$ | $83.0 \pm 2.3^{b}$ |
| | *Curcuma mangga* | $62.7 \pm 3.6^{b}$ | $65.5 \pm 3.1^{c}$ | $65.5 \pm 3.1^{c,d}$ | $75.2 \pm 3.7^{a}$ | $93.5 \pm 5.2^{a}$ | $100.0 \pm 0.0^{a}$ |
| | *Dehaasia longipedicellata* | $67.2 \pm 2.7^{a}$ | $68.5 \pm 4.3^{c}$ | $69.1 \pm 3.2^{c}$ | $50.0 \pm 2.8^{c}$ | $53.7 \pm 2.4^{d}$ | $53.7 \pm 2.4^{d}$ |
| | *Mitragyna speciosa* | $69.2 \pm 2.5^{a}$ | $69.2 \pm 2.5^{c}$ | $69.2 \pm 2.5^{c}$ | $42.0 \pm 3.6^{c}$ | $41.2 \pm 4.5^{e}$ | $42.0 \pm 2.2^{e}$ |
| | *Piper sarmentosum* | $62.5 \pm 3.5^{b}$ | $66.5 \pm 3.2^{c}$ | $94.7 \pm 3.8^{a}$ | $63.5 \pm 2.3^{b}$ | $82.0 \pm 1.9^{b}$ | $84.2 \pm 2.7^{b}$ |
| DCM | *Chisocheton erythrocarpus* | $65.8 \pm 4.2^{a,b}$ | $65.0 \pm 1.0^{c}$ | $83.5 \pm 1.5^{b}$ | $36.7 \pm 2.3^{d}$ | $37.7 \pm 5.2^{e}$ | $37.7 \pm 2.3^{e}$ |
| | *Cryptocarya nigra* | $56.5 \pm 5.1^{b,c}$ | $97.2 \pm 2.2^{a}$ | $97.2 \pm 4.1^{a}$ | $60.2 \pm 2.6^{b}$ | $84.7 \pm 2.0^{a,b}$ | $84.7 \pm 2.0^{b}$ |
| | *Curcuma purpurascens* | $57.6 \pm 3.3^{b}$ | $57.6 \pm 4.3^{d}$ | $57.6 \pm 1.5^{e}$ | $42.0 \pm 0.9^{c}$ | $62.7 \pm 2.7^{c}$ | $69.7 \pm 2.8^{c}$ |
| Hexane | *Alpinia conchigera* | $66.8 \pm 2.8^{a}$ | $100.0 \pm 0.0^{a}$ | | $57.6 \pm 3.9^{b}$ | $65.7 \pm 1.5^{c}$ | $98.0 \pm 1.4^{a}$ |
| | *Alpinia scabra* | $74.2 \pm 2.3^{a}$ | $96.8 \pm 4.7^{a,b}$ | $96.0 \pm 3.1^{a}$ | $63.0 \pm 1.3^{b}$ | $64.7 \pm 2.8^{c}$ | $67.5 \pm 3.1^{c}$ |
| | *Curcuma mangga* | $76.5 \pm 3.3^{a}$ | $92.7 \pm 3.3^{b}$ | $92.7 \pm 4.7^{a}$ | $53.2 \pm 2.6^{c}$ | $53.2 \pm 2.6^{d}$ | $53.2 \pm 2.6^{d}$ |
| | *Curcuma purpurascens* | $70.2 \pm 2.6^{a}$ | $75.2 \pm 2.1^{c}$ | $75.2 \pm 2.1^{c}$ | $58.7 \pm 4.2^{b}$ | $62.0 \pm 3.3^{c}$ | $62.0 \pm 3.3^{c}$ |
| | *Curcuma zedoaria* | $53.7 \pm 3.5^{c}$ | $64.2 \pm 2.1^{c}$ | $65.7 \pm 0.9^{c,d}$ | $55.9 \pm 3.2^{b,c}$ | $62.2 \pm 2.9^{c}$ | $64.3 \pm 4.1^{c}$ |
| | *Goniothalamus tapisoides* | $73.0 \pm 4.3^{a}$ | $100.0 \pm 0.0^{a}$ | | $71.5 \pm 2.2^{a}$ | $87.2 \pm 3.7^{a}$ | $96.2 \pm 3.6^{a}$ |
| | *Piper sarmentosum* | $60.5 \pm 2.3^{b}$ | $69.0 \pm 4.5^{c}$ | $98.3 \pm 1.3^{a}$ | $69.7 \pm 2.4^{a}$ | $79.0 \pm 2.0^{b}$ | $100.0 \pm 0.0^{a}$ |

*Note*. *Results expressed as the mean of four replicates, each set with 20 adults ($n$ = 80). Mean values within the same column annotated with the same letter in the superscript are not significantly different ($p \leq 0.05$)

### Contact Toxicity

Contact toxicity was performed by direct contact application on both insects with plant extracts. MeOH extracts of *C. aeruginosa* and *C. mangga*, DCM extract of *C. nigra*, and hexane extracts of *A. conchigera*, *C. mangga*, *C. purpurascens*, and *C. zedoaria* showed 100% mortality against *S. oryzae* adults within 28 days (Table 5). Results of the plant extracts against *R. dominica* adults followed the same trend except for hexane extracts of *A. conchigera* and *C. zedoaria*, which showed a maximum of 80% mortality within the experimental period. Similar to the previous test where *R. dominica* was more resistant to extracts as compared to *S. oryzae* ($x^2 = 22.81$, $p \leq 0.0001$).

### Antifeedant Activity

The antifeedant activity was carried out to investigate the plant extracts' ability to inhibit the insects' normal feeding behavior. In the antifeedant activity study, extracts with more than 70% feeding inhibition for both insects at a concentration of 5.0 mg/mL (MeOH extracts of *A. angustifolia* and *C. aeruginosa*, DCM extract of *C. nigra*, hexane extracts of *A. conchigera*, *A. scabra*, *C. manga*, and *C. purpurascens*) were further chosen to be examined for their inhibitory effect at the concentrations of 2.5 and 1.5 mg/mL. At the concentration of 2.5 mg/mL, methanol extracts of *C. aeruginosa* and hexane extracts of *A. conchigera* and *C. mangga* showed more than 90% feeding inhibition on both insects. Besides, the results indicated that the MeOH extracts of *C. aeruginosa*, hexane extracts of *A. conchigera*, *C. manga*, and *C. purpurascens* had higher antifeedant activity on both insects, although *R. dominica* is more resistant at the concentration of 1.5 mg/mL ($x^2 = 14.4$, $p \leq 0.001$) (Table 6).

## DISCUSSION

Investigation of plant-derived extracts and phytochemicals possessing biological activities and medicinal properties for pest control has significantly increased in recent years due to their selective toxicity on insect pests with little or no such effects on non-targeted organisms, the environment, and human health. It is now well established that the complex mixture of secondary metabolites contributes to plant extracts' activity even against some resistant strains of insect pests (Ahn et al., 1998; Akhtar et al., 2007; Anuradha et al., 2010; Zoubiri & Baaliouamer, 2014). In many cases, plant products have successfully combat various stored-product insect pests (Rajendran & Sriranjini, 2008; Shaaya et al., 1997). However, a study by Kim et al. (2003) showed that the susceptibility of a particular storage insect pest could differ from different plant extracts. Besides, a study revealed that *S. oryzae* adults were more susceptible to certain plant extracts as compared to *R. dominica* and the period of exposure interval is more important compared to the dosage of the extract (El-Nahal et al., 1989). In search for potent biopesticides, Jacobson (1989) identified several families, including Annonaceae, Asteraceae, Canellaceae, Lamiaceae, Meliaceae, and Rutaceae, with promising insecticidal activity, while

Table 5

*Insecticidal activities of the plant extract against* S. oryzae *and* R. dominica *adults, using the filter paper diffusion method, exposed to 5.41 mg/cm²*

| Extract | Plant[x] | Mean mortality (%) against *S. oryzae* after days of exposure[y,z] | | | | Mean mortality (%) against *R. dominica* after days of exposure[y,z] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 7th | 14th | 21st | 28th | 7th | 14th | 21st | 28th |
| Control | - | 00.0 ± 0.0[f] | 00.0 ± 0.0[e] | 1.2 ± 0.5[e] | 1.4 ± 0.5[d] | 00.0 ± 0.0[f] | 00.0 ± 0.0[f] | 00.0 ± 0.0[e] | 1.8 ± 0.4[e] |
| MeOH | *Curcuma aeruginosa* | 100.0 ± 0.0[a] | | | | 87.0 ± 4.4[a] | 99.7 ± 0.2[a] | 100.0 ± 0.0[a] | |
| | *Curcuma manga* | 87.0 ± 5.3[b] | 100.0 ± 0.0[a] | | | 68.2 ± 3.7[b] | 74.5 ± 3.4[b] | 78.5 ± 3.1[b] | 100.0 ± 0.0[a] |
| | *Mitragyna speciose* | 22.2 ± 5.4[d] | 22.2 ± 5.4[d] | 26.2 ± 4.1[d] | 27.7 ± 4.7[c] | 21.7 ± 4.9[d] | 32.7 ± 2.3[d] | 32.7 ± 2.3[d] | 33.5 ± 4.7[d] |
| DCM | *Cryptocarya nigra* | 84.5 ± 3.5[b] | 100.0 ± 0.0[a] | | | 80.5 ± 5.5[a] | 100.0 ± 0.0[a] | | |
| Hexane | *Alpinia conchigera* | 93.5 ± 2.7[b] | 94.2 ± 2.3[b] | 94.2 ± 2.3[b] | 100.0 ± 0.0[a] | 35.2 ± 2.7[c] | 77.2 ± 4.2[b] | 79.2 ± 3.3[b] | 79.2 ± 3.3[b] |
| | *Alpinia scabra* | 39.5 ± 2.7[c] | 56.7 ± 4.3[c] | 56.7 ± 4.3[c] | 56.7 ± 4.3[b] | 46.0 ± 3.2[c] | 59.5 ± 3.3[c] | 59.5 ± 3.3[c] | 59.5 ± 3.3[c] |
| | *Curcuma manga* | 100.0 ± 0.0[a] | | | | 82.7 ± 4.5[a] | 100 ± 0.0[a] | | |
| | *Curcuma purpurascens* | 100.0 ± 0.0[a] | | | | 88.7 ± 3.6[a] | 100 ± 0.0[a] | | |
| | *Curcuma zedoaria* | 90.0 ± 4.6[b] | 90.7 ± 4.4[b] | 100.0 ± 0.0[a] | | 62.7 ± 2.8[b] | 72 ± 3.3[b] | 80.2 ± 1.8[b] | 80.2 ± 1.8[b] |
| | *Goniothalamus tapisoides* | 9.5 ± 3.3[e] | 31.7 ± 2.0[d] | 32.8 ± 2.5[d] | 32.8 ± 2.5[c] | 5.7 ± 1.7[e] | 26 ± 3.9[e] | 33.7 ± 2.9[d] | 38.5 ± 3.8[d] |

*Note*.
[x]The plant extracts which did not show contact toxicity within 14 days were not included
[y]Each column represented the mean of four replicates, each set up with 20 adults ($n$ = 80)
[z]Means within a column followed by the same letter are not significantly different ($p \leq 0.05$)

Table 6

*Antifeedant activity of the active extracts against* S. oryzae *and* R. dominica *adults after 28 days of exposure*

| Extract | Plant[x] | Antifeedant index (%) mean ± SE[w] of *S. oryzae*[y,z] | | | Antifeedant index (%) mean ± SE[w] of *R. dominica*[y,z] | | |
|---|---|---|---|---|---|---|---|
| | | 5.0 mg/mL | 2.5 mg/mL | 1.5 mg/mL | 5.0 mg/mL | 2.5 mg/mL | 1.5 mg/mL |
| MeOH | *Alstonia angustifolia* | $100.0 \pm 0.0^{a}$ | $77.2 \pm 4.6^{b,c}$ | $31.6 \pm 2.8^{d}$ | $99.2 \pm 0.4^{a}$ | $86.7 \pm 2.9^{b}$ | $49.2 \pm 5.9^{b}$ |
| | *Curcuma aeruginosa* | $97.2 \pm 1.5^{a}$ | $94.7 \pm 1.2^{a}$ | $72.5 \pm 3.5^{b}$ | $100.0 \pm 0.0^{a}$ | $100.0 \pm 0.0^{a}$ | $67.5 \pm 3.6^{a}$ |
| | *Curcuma mangga* | $59.2 \pm 3.8^{e}$ | | | $69.3 \pm 4.0^{c}$ | | |
| | *Dehaasia longipedicellata* | $34.3 \pm 3.3^{g}$ | | | $20.5 \pm 3.1^{f}$ | | |
| | *Mitragyna speciosa* | $47.7 \pm 3.7^{f}$ | | | $39.7 \pm 4.8^{d}$ | | |
| | *Piper sarmentosum* | $12.0 \pm 3.3^{i}$ | | | $16.0 \pm 3.8^{f}$ | | |
| DCM | *Cryptocarya nigra* | $88.5 \pm 3.4^{c}$ | $75.7 \pm 3.3^{c}$ | $55.9 \pm 4.5^{c}$ | $93.0 \pm 2.5^{b}$ | $88.7 \pm 3.9^{b}$ | $45.7 \pm 3.9^{b}$ |
| | Curcuma purpurascens | $30.0 \pm 6.5^{g}$ | | | $13.5 \pm 3.7^{g}$ | | |
| | *Phoebe grandis* | $11.2 \pm 3.2^{i}$ | | | $22.5 \pm 3.9^{e,f}$ | | |
| Hexane | *Alpinia conchigera* | $100.0 \pm 0.0^{a}$ | $93.7 \pm 2.4^{a}$ | $82.5 \pm 4.1^{a}$ | $93.2 \pm 2.2^{b}$ | $82.9 \pm 2.8^{b,c}$ | $33.7 \pm 4.0^{c}$ |
| | Alpinia scabra | $79.5 \pm 4.1^{d}$ | $42.1 \pm 3.6^{d}$ | $8.5 \pm 2.2^{e}$ | $71.0 \pm 3.9^{c}$ | $44.6 \pm 2.8^{d}$ | $11.4 \pm 5.1^{d}$ |
| | *Curcuma mangga* | $100.0 \pm 0.0^{a}$ | $93.0 \pm 2.8^{a}$ | $81.4 \pm 4.8^{a}$ | $100.0 \pm 0.0^{a}$ | $100.0 \pm 0.0^{a}$ | $47.5 \pm 5.9^{b}$ |
| | *Curcuma purpurascens* | $94.3 \pm 4.6^{b}$ | $80.7 \pm 2.8^{b}$ | $51.4 \pm 3.7^{c}$ | $100.0 \pm 0.0^{a}$ | $74.1 \pm 4.9^{c}$ | $9.2 \pm 2.2^{d}$ |
| | *Curcuma zedoaria* | $51.5 \pm 5.9^{e,f}$ | | | $47.0 \pm 4.6^{d}$ | | |
| | *Goniothalamus tapisoides* | $16.7 \pm 5.4^{h}$ | | | $19.0 \pm 5.0^{f}$ | | |
| | *Mesua elegans* | $20.7 \pm 2.1^{h}$ | | | $27.0 \pm 4.0^{e}$ | | |
| | *Piper sarmentosum* | $36.7 \pm 5.2^{g}$ | | | $41.2 \pm 4.6^{d}$ | | |

*Note*.
[w]Standard error of *S. oryzae* and *R. dominica*
[x]Plant extracts with ≥ 70% feeding inhibition at 5.0 mg/mL were further evaluated at lower concentrations (2.5 and 1.5 mg/cm$^2$)
[y]Each column represented the mean of four replicates, each set up with 20 adults ($n = 80$)
[z]Mean within a column followed by the same letter are not significantly different ($p \leq 0.05$)

Chaubey (2012a, 2012b) and Tripathi et al. (2002) found that insecticidal activity was more common with the plants from the family Zingiberaceae with potent insecticidal activity against both the pests (Sok et al., 2017).

In fact, in the present study, hexane, DCM, and MeOH extracts of 22 Malaysian plants from 8 plant families with medicinal or pharmacological backgrounds were screened for insecticidal activity against adults of *S. oryzae* and *R. dominica*. The insect species directly affected the insecticidal activity of active plant extracts and was proportional to the exposure time. It was found that several plants from Annonaceae, Piperaceae, and Rubiaceae have shown strong insecticidal activity against both adults of *S. oryzae* and *R. dominica* together with Zingiberaceae with more than 67% of mortality after 72 hrs of treatment (Table 2). For some plants, several extracts were found to be active, indicating that the activity is not confined to compounds of any specific polarity. For example, hexane and MeOH extracts of *C. mangga* exhibited strong insecticidal activity against both insects, suggesting the active compounds are non-polar and polar. In the case of *P. sarmentosum*, hexane and MeOH extracts showed strong activity against *S. oryzae*. However, only hexane extract was active against *R. dominica*, thus suggesting that active compounds can be of different polarity.

The exposure interval-based bioassay implicates the importance of time duration for the observed activity of the extracts (El-Nahal et al., 1989). In the present study, the active plant extracts' toxicity levels rose over time. Thus, the mortality of *S. oryzae* caused by MeOH extract of *P. sarmentosum*, DCM extracts of *C. nigra*, hexane extracts of *A. conchigera*, *A. scabra*, *C. mangga*, *G. tapiosides*, and *P. sarmentosum* reached up to 90–100% after 21 days of exposure at their respective $LC_{50}$ concentrations (Table 4). The mortality of *R. dominica* increased similarly when exposed to MeOH extracts of *C. aeruginosa*, *C. mangga*, *P. sarmentosum*, DCM extract of *C. nigra*, hexane extracts of *A. conchigera*, *G. tapiosides*, and *P. sarmentosum* over 21 days.

MeOH extracts of *C. aeruginosa*, hexane extracts of *C. mangga* and *C. purpurascens* had the strongest contact toxicity against *S. oryzae* within 7 days of exposure to 5.41 mg/cm$^2$. In addition, MeOH extracts of *C. mangga*, DCM extracts of *C. nigra*, and hexane extracts of *A. conchigera* and *C. zedoaria* could cause up to 100% mortality of *S. oryzae*; however, they needed longer exposure. For *R. dominica*, MeOH extracts of *C. aeruginosa* and *C. mangga*, DCM extract of *C. nigra*, and hexane extracts of *C. mangga* and *C. purpurascens* could also cause up to 100% mortality by exposure for 28 days. Furthermore, most of these plants are reported to possess cytotoxic or anti-androgenic compounds such as 1 'S-1'-acetoxychavicol acetate (*A. conchigera*) (Malek et al., 2011), germacrone (*C. aeruginosa*), (*E*)-labda-8(17),12-dien-15,16-dial (*C. mangga*) (Ahmed Hamdi et al., 2014), and curcumenone (*C. zedoaria*) (Pan et al., 2016), which could be related to the toxicity towards the insects.

The use of antifeedant agents for pest control is considered superior over insecticidal agents as they inhibit insect feeding but do not directly kill insects and allow them to be available to their natural enemies (Arivoli & Tennyson, 2013; Frazier, 1986). Thus, using plant extracts with antifeedant activity holds promise in controlling insect resistance in storage pest management and helping maintain natural balance (Arivoli & Tennyson, 2013). In this study, MeOH extracts of *A. angustifolia* and *C. aeruginosa*, DCM extract of *C. nigra*, hexane extracts of *A. conchigera*, *A. scabra*, *C. manga*, and *C. purpurascens* showed potent antifeedant activity (more than 70% of the antifeedant index) against both pests at a concentration of 5.0 and 2.5 mg/mL. Therefore, these plants hold considerable potential for controlling *S. oryzae* and *R. dominica* populations in stored products. Comparatively, MeOH extracts of *C. aeruginosa* and hexane extracts of *A. conchigera* and *C. mangga* were the most potent feeding deterrent against *S. oryzae*, with 70% inhibition even at the concentration of 1.5 mg/mL. The extracts mentioned above were relatively potent against *R. dominica*, although they were more resistant than *S. oryzae*. Previous studies have shown alkaloids and terpenoids are the most potent insect antifeedant (Arivoli & Tennyson, 2013; Perry & Metzger, 1980; Schoonhoven, 1982) where *A. angustifolia* (Nasrullah et al., 2013; Raja et al., 2013) and *C. nigra* (Sun et al., 2017) are rich in alkaloid content, while *Curcuma* species (Sun et al., 2017) contain many terpenoid types of compounds. Besides, Zingiberaceae species have been reported to possess antifeedant activity (Dadang et al., 1998).

Hexane extracts, particularly from the plants of the Zingiberaceae family, contain a high percentage of volatile compounds (Pan et al., 2016; Sukari et al., 2008), which are suitable for fumigation against stored-product insects. A recent study of High-performance Liquid Chromatography (HPLC) analysis performed by Iskandar et al. (2021) on goniothalamin (GTN) compound from *G. tapiosides* found that the intensity and peak area of GTN in hexane extract was found to be the highest compared to other solvents extracts. Previous studies have shown that some plant extracts are useful in managing coleopterous insects (for example, *S. oryzae* and *R. dominica*) because of their fumigation ability in addition to their probable slow toxic effect minimizing the rate and frequency of pesticide application (Ahn et al., 1998; Kim et al., 2012). In addition, plant extracts with a rapid or slow mode of action can also be very useful in protecting stored products through the successful harmonizing of their immediate and late effects (Schmutterer, 1992, as cited in Sharma et al., 2014, pg.100).

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## CONFLICT OF INTEREST

The authors declare no conflict of interest in the preparation of this manuscript.

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# High Performance of Bacterial Strain Isolated from Bio-Extract for Cellulose Production

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## ABSTRACT

Bacterial cellulose (BC) producing bacterial strains were isolated from bio-extract (BE). Nine isolates that can produce BC in Hestrin–Schramm medium (HS medium) were identified. The BC production of these isolates was then investigated using agricultural waste as a raw material. The agricultural waste (banana, papaya, dragon fruit, and mango peels) was used as a carbon source for BC production. After incubation, the highest dry weight of BC reached 0.93±0.27 g/L, and 4.07±0.27 g/L was obtained from isolate BE073 in a medium containing mango and dragon fruit peels because the raw materials state is appropriate for bacterial growth. In a medium with papaya peel, the highest dry weight of BC was obtained from isolate BE052 at about 1.08±0.05 g/L. None of the strains was able to grow with the banana medium. However, all the isolate strains could grow and produce BC in the HS medium. The maximum dry weights of BC of 4.31±0.45 g/L, 4.23±0.13 g/L, and 4.21±0.25 g/L were obtained from isolates BE123, BE052, and BE073, respectively, and *Acetobacter xylinum* produced BC at 2.39±0.11 g/L. The structure and physical properties of BC produced from bacterial isolates using agricultural waste were characterized. It was similar to BC produced from HS medium and production from the reference strain *A. xylinum*. This study demonstrates the ability for BC production of bacterial strains isolated from bio-extract. It is also demonstrated that agricultural waste is a suitable and alternative carbon source for raw material in BC production.

*Keywords*: Agricultural waste, bacterial cellulose, bio-extract, isolation, local microorganism

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## INTRODUCTION

Bio-extract (BE) is a liquid product often applied in agriculture. It is used as a biofertilizer to replace chemical fertilizers and promotes plant growth (Kamla et al., 2007). In general, BE is produced from agricultural products or waste, such as fruits and vegetables, by local microorganisms through the fermentation process (Ali et al., 2021; Godlewska et al., 2021). Organic extracts will become increasingly important to agricultural systems because they are environmentally friendly, increase yields (Chutichudet & Chutichudet, 2022; Kamla et al., 2008), decrease the waste of agricultural products, and reduce agricultural investment costs. In addition, organic extracts support agricultural production by providing a nutrient supplement to the plant growth medium as a pure nutrient solution (Pathanapibul, 2003).

Based on the relationship between the fermentation of the BE and the local microorganisms in the raw material, the raw material can be fermented to form a blended liquid BE that produces a fiber sheet floating on the surface of the liquid (Bodea et al., 2022; Lemnaru et al., 2020). The fiber is thought to be bacterial cellulose (BC) produced from local microorganisms. BC has some unique properties. It is a pure polymer, is lignin and hemicellulose-free, temperature stable, and has a high water-holding capacity and swell ratio. These properties of BC make it suitable for applications in biomedical engineering products and for producing leatherette (Czaja et al., 2006; Fontana et al., 1990). It may also be used to produce dental crowns, food, bioelectronics, or biofilms (Esa et al., 2014), and it is also a component of emulsions in cosmetic products.

In local communities in Thailand, BE and BC are currently produced using traditional techniques, and local by-products or wastes are used as raw materials to provide a source of energy and nutrients for bacteria in BE production (Hadj Saadoun et al., 2021; Pandit et al., 2021). Villagers rely on BE to produce bacterial cellulose film, and this research has a large impact because the bacterial strain is a very important factor in producing BC. This research aims to use local microorganisms to study the efficiency of BC production. It aims to develop guidelines for using local microorganisms in the community to produce high-value biopolymers utilizing unused community agricultural waste, such as fruit peels, as a food source or carbon source for microbes. It will add value to agricultural waste and reduce production costs by utilizing high-value nutrients, leading to greater awareness among people in the community about the importance of conserving natural resources.

## MATERIALS AND METHODS

### Isolation of BC Producing Strains from BE

The BE samples were produced from various raw materials, including mangosteen, mango, *Tinospora cordifolia*, yacon, and banana stem by a farmer group in Nongseang Subdistrict, Pakplee District, Nakhon Nayok Province, Thailand, and the particularities of BC in BE of community present in

Figure 1. The BE samples were collected, and 10 mL was transferred into 100 mL of Hestrin–Schramm medium (HS medium) described by Hestrin and Schramm (1954). The HS medium contained 20 grams (g) of glucose (SCHARLAU, Spain), 5 g of peptone (SCHARLAU, Spain), 5 g of yeast extract (SCHARLAU, Spain), 2.7 g of disodium hydrogen phosphate ($Na_2HPO_4$) (QRëC, New Zealand), and 1.15 g of citric acid (SCHARLAU, Spain) per 1 liter (L) of media. The pH of the medium was adjusted to 4.2, and the samples were incubated at 30 °C for 3–4 days. The culture was then transferred again into HS agar by pour plate techniques and incubated at 30 °C for 7 days. Finally, a loopful of each colony successfully grown on HS agar was inoculated into 10 mL of HS medium. These isolate tubes were incubated at 30 °C for 4 days. After that, only isolated tubes with a BC covering on the surface of the medium were retained.

A single colony of bacteria was streaked on the medium. This single colony was then evaluated for its capability for BC production by transferring a loopful of the sample into a tube containing 10 mL of HS medium. Finally, the morphology was checked by Gram staining methods has 4 steps: (1) applying a primary stain by crystal violet, (2) adding a mordant by gram's iodine, (3) a rapid decolorization by ethanol, and (4) counterstaining with safranin (Smith & Hussey, 2005). Sequencing service provider Biodiversity Research Centre investigated the 16S rRNA sequencing analysis under the control of the Thailand Institute of Scientific and Technological Research.

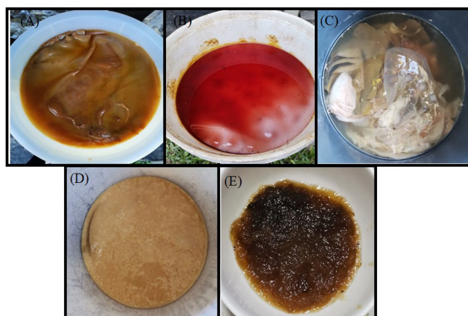


*Figure 1*. The BE was produced from different raw materials, including (A)*Tinospora cordifolia*, (B) mangosteen, (C) banana stem, (D) mango, and (E) yacon

### Preparation of Inoculums

*Acetobacter xylinum* was used as the reference strain. This strain was obtained from the Thailand Institute of Scientific and Technological Research (TISTR), Thailand. The strains isolated from the BE and *A. xylinum* were transferred to the HS medium and incubated at 30 °C for 3 days. The cultures were then used as initial inoculums for BC production.

### Production of BC Using Agricultural Wastes as a Carbon Source

The BC production was conducted using agricultural waste, such as fruit peels, as a carbon source. Banana peel, papaya peel, dragon fruit peel, and mango peel (Figure 2) were used for this study. The fruit peel was cut into small pieces and then blended in a blender until smooth, as shown in Figure 2, in preparation for its use as a carbon source in BC production.

*Figure 2*. The fruit peel samples after blending by blender: (A) mango peel, (B) papaya peel, (C) banana peel, and (D) dragon fruit peel

The BC production medium was prepared by adding 100 mL of water to 20% (w/v) fruit peel and 2% (w/v) of sucrose (SCHARLAU, Spain). The medium was then sterilized by autoclave at 110 °C for 15 min. Finally, 5% (v/v) of inoculum was inoculated and incubated at room temperature for 14 days. After incubation, the BC film was collected for analysis of the characteristics.

### BC Analysis Methods

The BC film was washed and soaked thoroughly with distilled water for 2–3 days. It was then boiled in 1% (w/v) sodium hydroxide solution (NaOH) (SCHARLAU, Spain) for 1 h and washed with distilled water until reaching a neutral pH of about 7.0. The wet weight of the BC film was measured. The samples were dried at 60 °C until constant weight and weighed for dry weight. The productivity of the BC films was determined by calculating their weight per fermentation day (d). The structure of the dried BC film was characterized using Fourier-transformed infrared (FTIR) spectroscopy and was scanned in the range of 4,000-400 $cm^{-1}$.

The moisture content of the BC films was calculated based on their water loss after drying. First, the dehydrated BC film was weighed to obtain an initial weight of the sample ($W_w$). Afterward, the sample was dried at 60 °C until reaching a constant weight and weighed for the dry weight ($W_d$). Finally, the water content was calculated according to the following equation:

$$\%\ \text{Moisture content} = \frac{(Ww-Wd)}{Ww} \times 100\%$$

## RESULTS AND DISCUSSION

### Strain Isolation and Identification

Bacteria isolated from the BE were grown and isolated on HS agar at 30 °C. After incubation, 19 isolates were observed to grow successfully on HS agar. The isolates' production of BC was investigated by inoculating a loopful of each colony into an HS medium. The results showed that 11 isolates exhibited the ability to produce BC (Table 1). It is important to identify the bacterial strain in the BE because different BE products have widely varying combinations of microbial strains (Mazzucotelli et al., 2013; Nishizawa et al., 2012).

Table 1
*Bacteria isolated from BE samples*

| Sample | Source of sample | Isolate code | Production of BC |
|---|---|---|---|
| 1 | BE from stem banana 1 | BE011 | X |
| 2 | BE from mango 1 | BE021 | ✓ |
| | | BE022 | ✓ |
| 3 | BE from *Tinospora cordifolia* 1 | BE031 | X |
| 4 | BE from *Tinospora cordifolia* 2 | BE041 | X |
| 5 | BE from heart-leaved moonseed 3 | BE051 | ✓ |
| | | BE052 | ✓ |
| | | BE053 | X |
| 6 | BE from stem banana 2 | BE061 | X |
| 7 | BE from mangosteen 1 | BE071 | ✓ |
| | | BE072 | ✓ |
| | | BE073 | ✓ |
| 8 | BE from mangosteen 2 | BE081 | X |
| 9 | BE from yacon 1 | BE091 | X |
| 10 | BE from yacon 2 | BE101 | X |
| 11 | BE from mango 2 | BE111 | ✓ |
| 12 | BE from yacon 3 | BE121 | ✓ |
| | | BE122 | ✓ |
| | | BE123 | ✓ |

*Note*. BE = Bio-extract; BC = Bacterial cellulose

The morphology and aggregation of cells were investigated by Gram staining methods (Smith & Hussey, 2005) using a light microscope, and the results are shown in Figure 3.

Isolated BE052, BE073, and BE123 were identified using the partial sequences 16S rRNA technique. It was found that all isolated strains revealed 99% similarity to *Komagataeibacter* spp.

**Agricultural Byproduct Fermentation to BC Production**

Bacterial strains isolated from BE were investigated for their ability to produce BC by a static fermentation process. The agricultural wastes consisting of mango peel, papaya peel, banana peel, and dragon fruit peel were used as a carbon source, and the growth of the isolated bacterial strains in the HS medium was compared. After incubation for 14 days, the results show that all the isolate strains could grow and produce

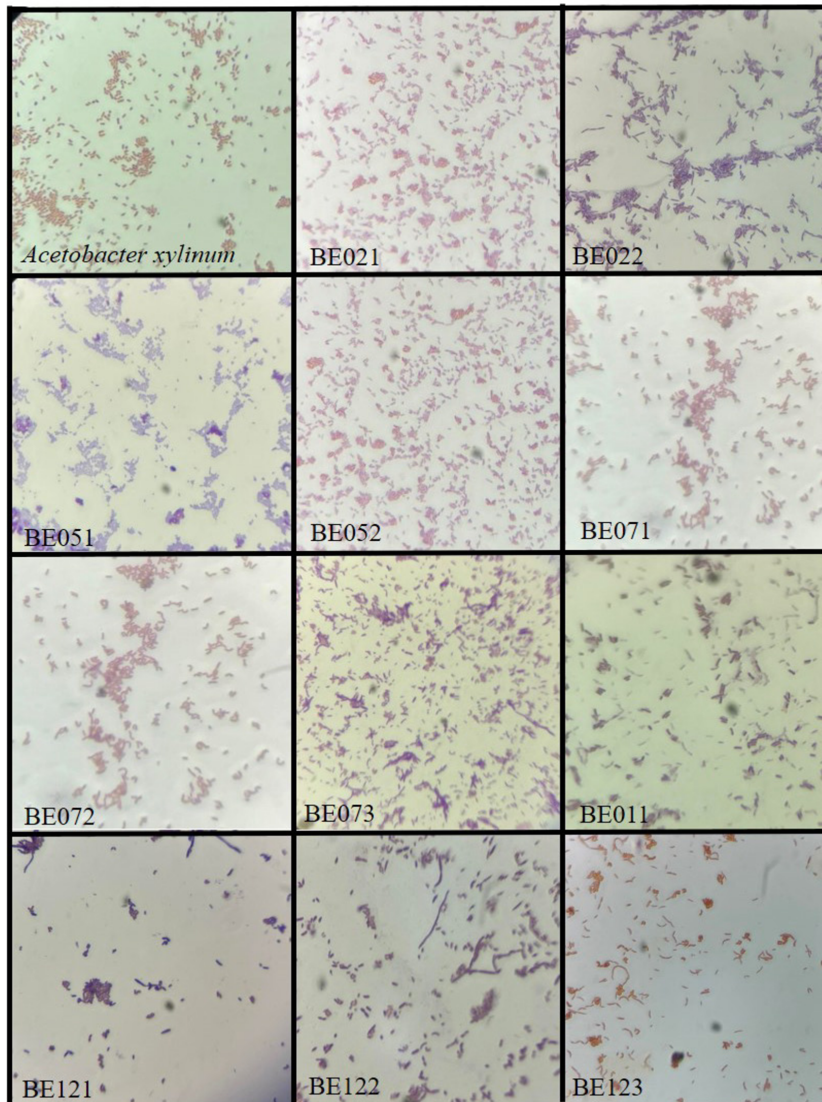
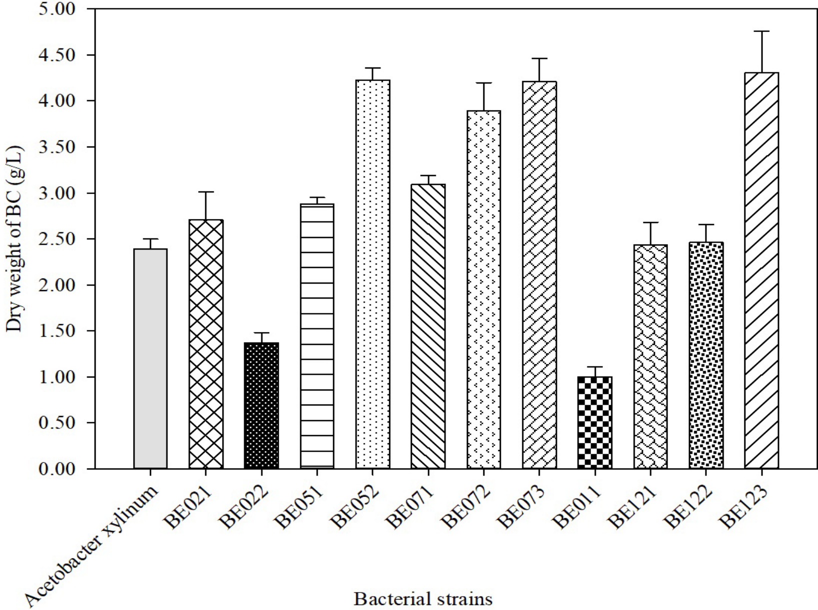
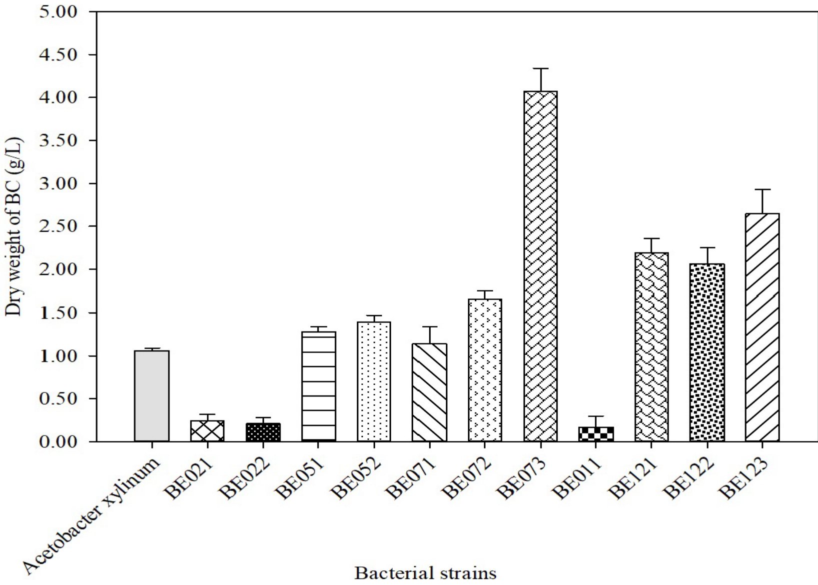



*Figure 3*. Photomicrograph (×100) of the isolated cells and the reference strain of *A. xylinum* by Gram staining method after 7 days of incubation

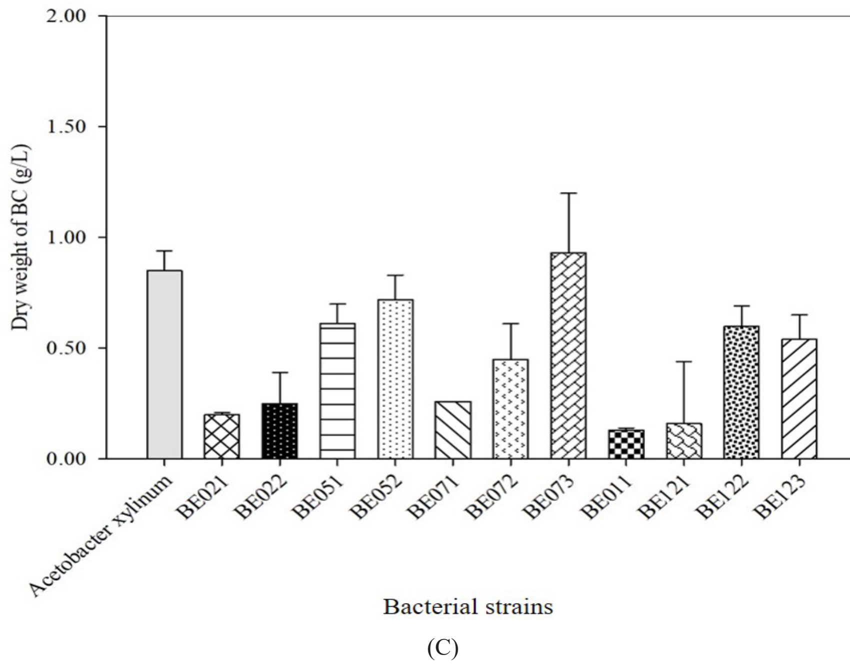
BC in the HS medium (See Figure 4A). The maximum dry weights of BC of 4.31±0.45 g/L, 4.23±0.13 g/L, and 4.21±0.25 g/L were obtained from the isolates BE123, BE052, and BE073, respectively, while *A. xylinum* produced BC at 2.39±0.11 g/L. In the media with mango peel and dragon fruit peel, the highest dry weights of BC were obtained from isolate BE073 and were 0.93±0.27 g/L and 4.07±0.27 g/L, respectively. For the medium with papaya peel, the highest dry weight of BC was obtained from isolate BE052 and was about 1.08±0.05 g/L. The results are shown in Figure 4.




(A)




(B)

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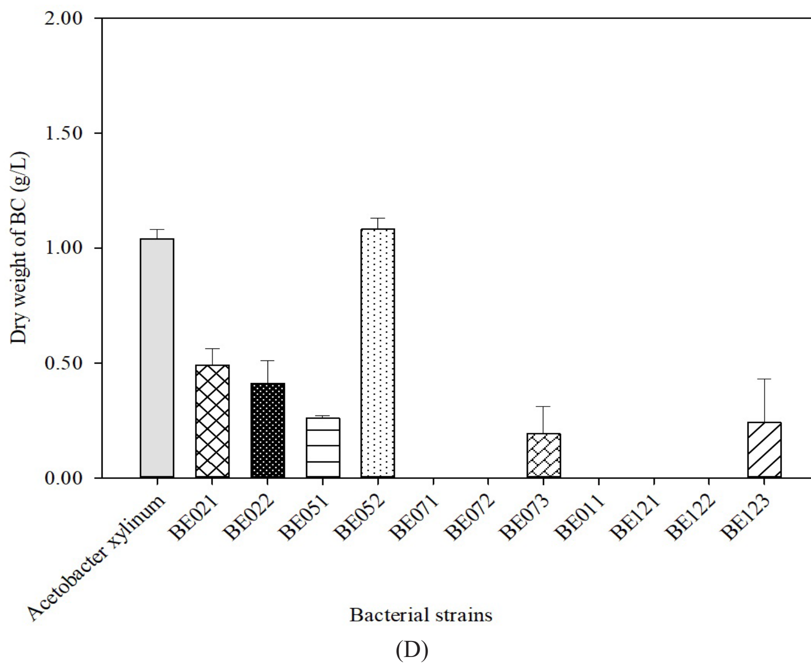


(D)

*Figure 4*. BC produced by *A. xylinum* and bacteria strains in (A) HS medium containing, (B) dragon fruit peel, (C) mango peel, and (D) papaya peel

It was observed that three bacterial isolates grew very well in the HS medium and produced high yields of BC above those of an agricultural waste product using a bacterial carbon source. This higher productivity from the bacterial isolate results in producing a fiber using available sucrose and a nitrogen and carbon source (Brückner & Titgemeyer, 2002; Molina-Ramírez et al., 2017). However, the BC developed in the HS medium differs from that produced by traditional village techniques because the microbes are supplied with sugar from decomposition by microbial enzymes in the absence of oxygen (Boopathy et al., 2001; Ishikawa, 1928). Therefore, processing affects the development of microbes.

Bacteria were found in the banana peel medium but did not produce BC. It was observed during the research that the banana peel became very dry after preparation, and the high fiber but low sugar conditions were possibly unsuitable for bacterial BC production. In contrast, some bacterial growth was found in the papaya peel medium, but there was a low production of BC because, in the papaya peel, there is low sugar, and the sugar in the papaya peel of less than 7.8 g per kg (United States Department of Agriculture [USDA], 2019) below the average HS medium 20 grams of sugar per liter. However, it contains other components, such as minerals and pectin (Mavani et al., 2020; Rojas-Flores et al., 2021), which allow the microorganisms to produce BC.

The color of the BC varies based on the color characteristics of the medium. For example, it varies between cinnamon, yellow, and brown because the BC fibrils absorb the medium pigments (Kim & Kim, 2022; Shim & Kim, 2018). However, the BC can be cleaned by boiling it in an alkaline solution and soaking it in deionized water until the pH is neutral. The resulting BC film color is shown in Figure 5.

The productivity observed for the isolated bacteria that produced the high BC in different media was compared with the pure bacterial strain *A. xylinum*. The bacterial strains found by isolation from BE had BC production about 104–308% greater than the pure bacterial strain *A. xylinum*, as shown in Table 2. Therefore, the results demonstrate the potential of BC production in an industrial setting.

The moisture content is related to the water-holding capacity of BC, and the moisture content observed in this study ranged between 76% to 91%, which is lower than the generally observed moisture of BC of 97–99% (Rebelo et al., 2018). However, the water-holding ratio is more significant as water absorption is the most important consideration in developing future production methods (Ul-Islam et al., 2012).

The current experiment uses fruit peel, including the peel of mango, dragon fruit, and papaya, to produce BC, demonstrating the potential to use a carbon source to produce BC. In addition, the bacteria isolated from BE are also available to grow and produce BC. The potential BC production from BE compared with other carbon sources from agricultural waste products is shown in Table 3.

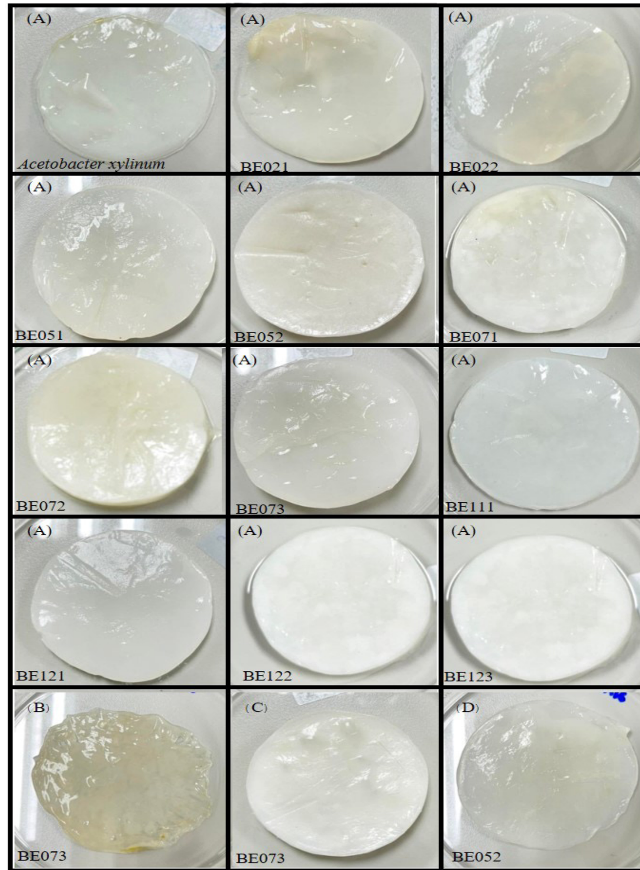


*Figure 5*. BC films produced from *A. xylinum* and isolated bacteria in (A) HS medium, (B) media with papaya peel, (C) dragon fruit peel, and (D) mango peel

Table 2

*The productivity of bacterial strain in medium*

| Medium | Bacterial strain | Productivity (g/L/d) | % Moisture content |
|---|---|---|---|
| HS medium | *Acetobacter xylinum* | 0.171±0.008 | 82.40±1.86 |
| | Isolate BE123 | 0.308±0.032 | 81.96±0.82 |
| Mango peel | *Acetobacter xylinum* | 0.061±0.007 | 91.68±1.80 |
| | Isolate BE073 | 0.067±0.019 | 84.03±1.16 |
| Dragon fruit peel | *Acetobacter xylinum* | 0.076±0.002 | 89.53±0.03 |
| | Isolate BE073 | 0.291±0.019 | 76.01±0.27 |
| Papaya peel | *Acetobacter xylinum* | 0.074±0.003 | 88.29±0.24 |
| | Isolate BE052 | 0.077±0.004 | 88.50±0.56 |

*Note*. Productivity = BC production per fermentation time

Table 3
*Bacterial strains and BC production in different fruit wastes*

| Carbon source | Bacterial strain | BC production (g/L) | Fermentation time (Days) | References |
|---|---|---|---|---|
| Mango peel | Isolate BE073 | 0.93 | 14 | Present study |
| | *Acetobacter xylinum* | 0.85 | 14 | |
| Dragon fruit peel | Isolate BE073 | 3.74 | 14 | |
| | *Acetobacter xylinum* | 1.06 | 14 | |
| Papaya peel | Isolate BE052 | 1.08 | 14 | |
| | *Acetobacter xylinum* | 1.04 | 14 | |
| Pineapple and watermelon peels | *Komagataeibacter hansenii* | 30 (Wet weight) | 7 | Kumbhar et al. (2015) |
| Banana peel | *Komagataeibacter nataicola* | 0.89 | 9 | Moukamnerd et al. (2020) |
| Passion fruit peel | | 0.31 | 9 | |
| Lemon peel | *Komagataeibacter sucrofermentans* | 5.20 | 13 | Andritsou et al. (2018) |
| Grapefruit peel | | 5.00 | 13 | |
| Orange peel | | 2.90 | 13 | |
| Orange peel | *Gluconoacetobacter xylinus* | 3.40 | 8 | Kuo et al. (2017) |

**The Structure of BC**

After collection of the BC samples from the media and drying at 60 °C, the structure of the BC samples was analyzed by FTIR spectrophotometer. The samples were scanned in the range of 4,000−400 cm$^{-1}$ to compare the BC structure to products from *A. xylinum* in the HS medium. The results are presented in Figure 6.

The FTIR spectra of BC obtained from bacteria isolated from BE in different fruit peels were similar to those obtained from a BE sample grown in an HS medium by *A. xylinum*. All the BC samples peaked in the 3600–3000 cm$^{-1}$, corresponding to the hydroxyl group (–OH) (Buldum et al., 2018; Huang et al., 2010). The spectra peaks normalized to 900 cm$^{-1}$ and ~ 1249 cm$^{-1}$ were attributed to the carbon-oxygen bond (C−O) and carbon-oxygen-carbon bond (C−O−C) stretching within glucose (Carrillo et al., 2004; Wong et al., 2009). The 1330−1495 cm$^{-1}$ corresponds to the hydrogen-carbon-hydrogen bond (H–C–H) and oxygen-carbon-hydrogen bond (O–C–H) in-plane bending (Andritsou et al., 2018). In addition, the FTIR spectrogram showed results similar to those obtained from a previous study reporting on BC produced from a pure bacterial strain (Hirai et al., 1998).

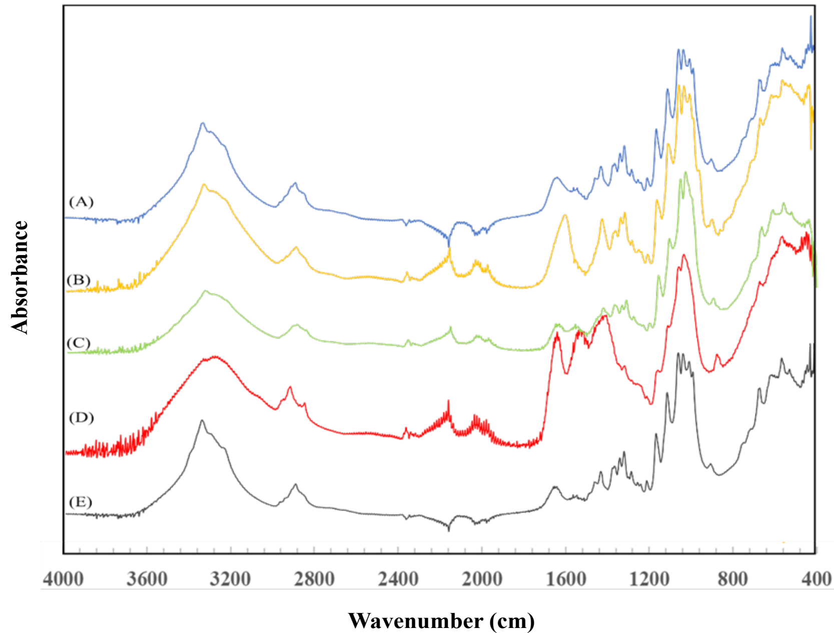


*Figure 6*. FTIR spectra: BC obtained from isolate BE052 in (A) papaya peel, (B) isolate BE073 in dragon fruit peel, (C) isolate BE073 in mango peel, (D) isolate BE123 in HS medium, and (E) *A. xylinum* in HS medium

## CONCLUSION

The 11 bacterial isolates were obtained from BE and demonstrated that the microbial components of BE are capable of BC production. They could grow and produce BC in the HS medium, and their yield of BC in the HS medium was 104–308% that of the pure bacterial strain *A. xylinum*. The agricultural waste in communities consisting of papaya peel, banana peel, and dragon fruit peel is a possible carbon source for BC production; however, this agricultural waste, such as banana peel that is low in moisture and bulky, may not be suitable for use in BC production. The color variation of BC is due to the color of the medium culture. The study found that BC water content ranges from 76% to 91% of its water-holding capacity. The structural analysis of BC revealed functional groups, including hydroxyl groups, C−O, and C−O−C stretching within the glucose compound. However, agricultural waste can be used as a source of alternative carbon and coupled with bacterial strains isolated from BE to increase BC production. The present finding could be benefited the community in utilizing the studies agricultural wastes for BC production.

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# Pertanika Journal of Tropical Agricultural Science

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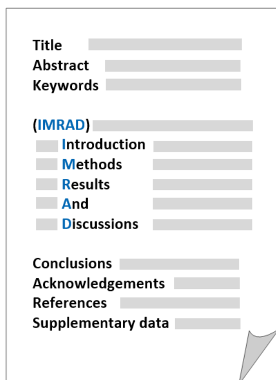


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