Pertanika Journal of

# TROPICAL AGRICULTURAL SCIENCE

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PERTANIKA JOURNALS

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*Pertanika* Journal of Tropical Agricultural Science
*Pertanika* Journal of Tropical Agricultural Science

# 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.

### *Mission*

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

### *Quality*

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

### *Abstracting and indexing of Pertanika*

*Pertanika* is now over 42 years old; this accumulated knowledge has resulted in Pertanika Journal of Tropical Agricultural Science being abstracted and indexed in SCOPUS (Elsevier), Clarivate Web of Science (ESCI), EBSCO, DOAJ, Agricola, ASEAN CITATION INDEX, ISC, Microsoft Academic, Google Scholar, National Agricultural Science (NAL), and MyCite.

### *Citing journal articles*

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

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

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***Lag time***

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Authors are not permitted to add or remove any names from the authorship provided at the time of initial submission without the consent of the journal's Chief Executive Editor.

***Manuscript preparation***

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).

***Editorial process***

Authors who complete any submission are notified with an acknowledgement containing a manuscript ID on receipt of a manuscript, and upon the editorial decision regarding publication.

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

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

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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In the peer review process, 2 or 3 referees independently evaluate the scientific quality of the submitted manuscripts. At least 2 referee reports are required to help make a decision.

Peer reviewers are experts chosen by journal editors to provide written assessment of the **strengths** and **weaknesses** of written research, with the aim of improving the reporting of research and identifying the most appropriate and highest quality material for the journal.

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

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

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

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

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

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

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

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

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## ABSTRACTING AND INDEXING OF PERTANIKA JOURNALS

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**Pertanika Journal of Tropical Agricultural Science**
**Vol. 45 (2) May. 2022**

## Contents

# Foreword

Welcome to the second 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 12 articles; a short communication and the rest are regular articles. The authors of these articles come from different countries namely Indonesia, Malaysia, and Thailand.

A selected article entitled "Zebrafish Embryotoxicity and Teratogenic Effects of *Christia vespertilionis* Leaf Extract" tested the toxic and teratogenic effects of the plant on the embryonic development of zebrafish (*Danio rerio*) as the animal model. The results showed that the methanolic leaf extract of *C. vespertilionis* is toxic to zebrafish embryos at concentrations of 200 µg/mL and above, which cause the multiple signs of developmental abnormalities. Hence, the extreme caution is advised in using the plant for healthcare purposes at uncontrolled concentrations. The further details of this study are found on 351.

Nur Azura Adam and her teammates from Universiti Putra Malaysia investigated the best artificial propagation technique for stingless bee *Heterotrigona itama*. Three different artificial propagation techniques, namely splitting, bridging, and splitting bridging, were studied for eight consecutive weeks. Honey pot quantity, colony division, and pollen pot quantity were observed and recorded weekly. It concluded that the splitting technique is the only successful artificial technique that obtained new brood cells and queen of *Heterotrigona itama*. Full information of this study is presented on 367.

A regular article entitled "Effect of Streptomyces Inoculation on *Ipomoea aquatica* and *Pachyrhizus erosus* Grown under Salinity and Low Water Irrigation Conditions" revealed that the salinity affected the success of plant growth-promoting bacteria used in *Ipomoea aquatica* and *Pachyrhizus erosus* cropping more than the water-limited effect. In other words, salinity was the most effective factor, and irrigation was the least influential factor on both plants' growth. The detailed information of this article is available on 411.

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

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

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

**Chief Executive Editor**

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# Zebrafish Embryotoxicity and Teratogenic Effects of *Christia vespertilionis* Leaf Extract

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## ABSTRACT

*Christia vespertilionis* or butterfly wings is a traditional medicinal plant used to treat, among others, colds and bronchitis. The plant was also reported to be a remedy for cancer, with several products based on the plant becoming commercially available, raising some safety concerns on its consumption. The present study was carried out to assess the toxic and teratogenic effects of the plant on the embryonic development of zebrafish (*Danio rerio*) as the animal model. Zebrafish embryos were exposed to 50, 100, 200, 400, and 800 µg/mL of the methanolic leaf extract of *C. vespertilionis*, starting from 5 to 120 hours post-fertilization (hpf). The median lethal concentration ($LC_{50}$) value of the extract was determined to be 419.84 µg/mL, which is within the safety limit stipulated by the Organisation for Economic Co-operation and Development (OECD) guideline. However, results from the teratogenicity evaluation revealed multiple signs of developmental defects in embryos exposed to 200 µg/mL and higher concentrations of the extract. The magnitude of the defects was observed to be concentration-dependent. Moreover, no hatching and spontaneous movement of tail coiling were observed at 400 and 800 µg/mL concentrations due to the delayed growth and early mortality, respectively. A significant reduction in heartbeat rate was also reported for the surviving embryos at the 400 µg/mL test concentration. The present study has provided preliminary results on the potentially toxic and teratogenic effects of the extract at high concentrations.

*Keywords*: *Christia vespertilionis*, embryotoxicity, methanolic extract, teratogenic effects

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## INTRODUCTION

Plants have served as a valuable source of chemical constituents with a broad spectrum of pharmacological properties, many of which have been translated into clinically used drugs (Ghasemzadeh et al., 2015). The promising potential of plants, especially those with a history of ethnomedicinal uses in curing various diseases and ailments, has also led to the growth of a wide variety of herbal products and supplements globally. However, despite the beneficial effects on human health, herbs and products derived from them have also been associated with cases of adverse side-effects resulting from their ingestion (Hussin, 2001). Thus, the toxicological assessment of herbal products is an essential step within the framework of herbal product development to protect and ensure consumer safety.

By convention, various mammalian models such as mice, rats, and rabbits have been widely used in toxicological studies (Caballero & Candiracci, 2018). Owing to the fact that the whole animal system is typically closely related to human toxicity, the use of animal models is considered a gold standard in toxicological testing (Jayasinghe & Jayawardena, 2019). However, in recent years, the use of zebrafish (*Danio rerio*) as an alternative to the classical higher vertebrate models has gained increasing attention. The wide usage of zebrafish is mainly attributed to its high genetic similarity to humans; zebrafish possess approximately 70% homology with humans, and about 84% of its genes appear to be related to human disease (Howe et al., 2013). Presently, compared to adult zebrafish, embryos are more increasingly being used for toxicological evaluations due to their optical transparency, which permits direct visualization of the model's developmental stages without a need for surgical procedure (Jayasinghe & Jayawardena, 2019). In addition, teratogenic effects upon exposure to chemical substances can be easily observed in zebrafish, giving the excellent predictive ability of the bioassay in evaluating developmental toxicity in mammals (Gao et al., 2014). Moreover, testing on the zebrafish model can also be completed in a short timeframe, which is extremely valuable, and the embryos exhibit a good dose-response to toxicity (Zhang et al., 2003).

*Christia vespertilionis*, popularly known as 'butterfly wing' or 'rerama,' is a plant of the *Christia* Moench genus in the Fabaceae family. This species is widespread in tropical Southeast Asia and exists in two varieties: red and green-leafed. Traditionally, *C. vespertilionis* has been reported to be used in treating colds, bronchitis, tuberculosis, muscle weakness, poor blood circulation, bone fractures, snake bites, and scabies (Dash, 2016). Pharmacological properties reported on the plant leaves included anti-proliferative (Hofer et al., 2013), cytotoxicity (Abd Latip & Abd Mutalib, 2019; Lee et al., 2020; Nguyen-Pouplin et al., 2007), antimalarial (Nguyen-Pouplin et al., 2007), antidiabetic (Murugesu et al., 2020), and antioxidant properties (Abd Latip & Abd Mutalib, 2019; Lee et al., 2020; Murugesu et al., 2020). Individual bioactive constituents

responsible for these biological properties have yet to be identified. However, in our molecular network-based dereplication of the chemical constituents of the plant, it is shown to be rich in flavonoids and phenolic acids (Norazhar et al., 2021).

In Malaysia, the green-leafed variety gained popularity in recent years due to testimonial reports on the therapeutic uses of the plant, which included as an herbal treatment for cancer. According to some patients diagnosed with cancer, consuming a water decoction of the fresh leaves of this plant helped in improving their health and claimed to have 'cured' their cancer (Zakaria, 2015). These have raised public concerns with respect to the validity of the efficacy claims and, more importantly, product safety. Previously, in a study by Nurul et al. (2018), although subacute oral administration of the ethanolic leaf extract to rats showed no mortality, mild to moderate lesions of hepatic necrosis and degeneration, and eventually hepatitis, were observed in all treated groups. Apart from this study, there were no other toxicity reports on the plant. Thus, there is still limited and inadequate toxicity and teratogenicity information on *C. vespertilionis* (green-leafed variety), emphasizing the need for more research to properly establish the toxicity profile of the plant and determine the safe levels for its practical usage for healthcare. The present study was thus carried out to address some aspects of this need by evaluating the toxic and teratogenic effects of the plant extract on the embryonic development of zebrafish (*Danio rerio*).

## MATERIALS AND METHODS

### Chemicals

Analytical grade methanol and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific, Malaysia.

### Plant Material

*Christia vespertilionis* (green-leafed variety) was obtained from a plant nursery in Skudai, Johor, Malaysia, and taxonomically authenticated by Dr. Mohd Firdaus Ismail, a botanist at the Institute of Bioscience, Universiti Putra Malaysia. A voucher specimen (MFI 0150/20) has been deposited in the herbarium unit of the Institute of Bioscience, Universiti Putra Malaysia, for the record.

### Extraction

Fresh leaves of *C. vespertilionis* were washed and dried in a circulating air oven at 40 ºC until constant weight. The dried leaves were then ground into a fine powder using a mechanical blender (HR2056, Philips, Netherlands). Next, 10 g of the ground leaves were mixed with 100% methanol at a solid to liquid ratio of 1:10 (*w/v*) and sonicated for 30 minutes under a frequency of 53 kHz and power of 100 W, bath temperature maintained between 30 ºC to 40 ºC. The extract was filtered with Whatman filter paper No.1 (GE Healthcare, USA), and the solvent was removed using a rotary evaporator under reduced pressure, with the temperature-controlled at 40 ºC. The crude extract was stored at -80 ºC freezer and further lyophilized using a Labconco®

FreeZone Freeze Drier System (USA). The freeze-dried extract was then stored in an airtight container at 4 ºC until further use.

**Fish Husbandry**

Fish experiments were carried out as approved by the UPM's Institutional Animal Care and Use Committee (IACUC), approval letter number UPM/IACUC/AUP-R045/2019. Adult zebrafish (AB strain), all (> six months old), were maintained under 10:14 h of the dark: light cycle with ambient temperature at 28.5 °C in 3 L aquarium tanks. The adult fishes were originally purchased from the Institute of Molecular and Cell Biology, Singapore. Then, they were maintained and propagated in Bioassay Unit, Natural Medicines and Products Research Laboratory (NaturMeds), IBS, UPM. Adult males and females used for this experiment belong to the F4 generation. Only five fish with a female to male ratio of 3:2 were placed per tank to ensure a stress-free environment for the highly sensitive fish. The tanks were continuously supplied with water by a recirculating water system. The fish were fed with brine shrimps (*Artemia salina*, San Francisco Bay Brand, USA) four times per day to ensure healthy and high fecundity. The volume of brine shrimps fed to the fish was approximately 4 mL/3 L tank for each feeding.

**Spawning, Collection, and Selection of Embryos**

Healthy (visual assessment of body condition scoring according to Clark et al., 2018), active, and well-fed adult zebrafish (> six months old) were selected for breeding. Five fishes were maintained in a 3 L aquarium equipped with a recirculation water system maintained under 10:14 h of the dark: light cycle at 28.5 °C, with a female to male ratio of 3:2. Artificial aquarium plants were placed in the spawning tank together with a spawn trap for egg collection to stimulate spawning. Three spawning tanks were set up for the experiment to have an adequate supply of fish eggs. Fertilization usually occurs in the morning, within 30 minutes after the light is turned on. Fish eggs were collected, washed with distilled water, rinsed with embryo media [15 mM sodium chloride (NaCl), 0.5 mM potassium chloride (KCl), 1 mM magnesium sulfate ($MgSO_4$), 0.15 mM monopotassium phosphate ($KH_2PO_4$), 0.05 mM disodium phosphate ($Na_2HPO_4$), 1 mM calcium chloride ($CaCl_2$), 0.7 mM sodium bicarbonate ($NaHCO_3$), pH 7.0], transferred into clean petri dishes containing embryo media (E3M), and incubated at 28 °C. According to the guideline by Organisation for Economic Co-operation and Development (OECD) (2013), the fertilization rate should be more than 50%, while in our laboratory standard protocol, the experiment will be conducted only when the rate of fertilization is more than 70%. At 4 hpf, normally fertilized embryos that reached the gastrulation stage (50% epiboly) were selected for this experiment. The selection was carried out by examining the collected eggs under a standard dissecting microscope (SZX-12, Olympus, Japan) with magnification set to 3x. The selected fertilized embryos were rinsed with E3M,

and any dead or unfertilized eggs were removed (to eliminate fungal growth).

**Embryonic Exposure Experiments**

The exposure experiment was performed in 24-well plates according to the method described in OECD (2013). After initial range-finding experiments, five concentrations (50, 100, 200, 400, 800 µg/mL) of the extract were selected as the final test concentrations. A stock solution was prepared by dissolving 0.05 g of the sample in 1000 µL DMSO. The highest treatment concentration (800 µg/mL) was first prepared by diluting 240 µL of the stock solution with 14,760 µL of E3M. From this concentration, two-fold serial dilutions were further made to give the subsequent treatment concentrations. The percentage of DMSO in the highest treatment concentration (800 µg/mL) was calculated to be 1.6%, which was well within the safe limit of the organic solvent allowed for zebrafish embryo assay (Maes et al., 2012). Ten embryos at the gastrulation phase were transferred into each well containing the different treatment concentrations. For the control group, embryos were exposed to 1.6% of DMSO in E3M. The maximum volume per well was kept to 2 mL. The plate was incubated at 28 °C for the exposure experiment. Three independent replicates were performed for each treatment concentration.

**Evaluation of Toxicity Effects.** A series of toxicity parameters such as mortality rate, spontaneous movement of tail coiling behaviour (at 24 hpf), heartbeat rate (at 48 hpf), and hatching rate (at 72 hpf). Upon completion of the early developmental process, a zebrafish larval is normally released from the chorion because of chorion breakdown. Normally, the hatching process is completed by 72 hpf; however, this biological process is interrupted in toxic conditions. The hatching rate was determined by quantifying the number of successfully hatched embryos at 72 hpf. All observations were made and recorded after viewing the embryos under a standard dissecting microscope (SZX-12, Olympus, Japan). The mortality rate data obtained was then used to determine the median lethal concentration ($LC_{50}$) of the extract by means of probit analysis (Finney, 1971) in Microsoft Excel. The number of tails coiling observed over one minute for the individual embryo was manually counted. The embryo was habituated for five minutes under the microscope before starting the tail coiling count. One complete cycle of coiling is represented by a full-body contraction that brings the tip of the tail to the head, which involves two alternating side to side contractions (left-right) (Saint-Amant & Drapeau, 1998). The heartbeat of the individual embryo was determined by manually counting the embryo's heartbeat over 1 minute. No anaesthetic drug was used while measuring the heartbeat.

**Evaluation of Teratogenic Effects.** Several parameters of teratogenicity such as the abnormal shape of head, eyes, and heart, bent body axis, growth retardation, uninflated

swim bladder, and deformity of yolk were assessed for 120 hours by viewing under a standard dissecting microscope (SZX-12, Olympus, Japan).

### Statistical Analysis

All results obtained were expressed as mean ± standard deviation (SD) from three independent replicates, calculated using Minitab software (Version 16, Minitab Inc., USA). In addition, the *P* values were obtained from analysis of variance (ANOVA) analysis using the post-hoc Tukey's test where *($P \leq 0.05$) was significantly different from the control group.

## RESULTS

**Effect on Mortality Rate.** The effect of the extract on zebrafish embryos mortality rate was evaluated over a range of concentrations (50-800 µg/mL). As shown in Figure 1, zero mortality was recorded for the control and low concentration groups (50 and 100 µg/mL). However, the mortality rate of the embryos was significantly increased with exposure to higher concentrations starting from 200 µg/mL, inducing a significant increment in mortality rate from 10% (200 µg/mL) to 100% (800 µg/mL). In particular, 200 and 400 µg/mL concentrations induced 10% and 50% mortality within 48 hpf, respectively. Meanwhile, it was observed that the highest test concentration of 800 µg/mL induced 56% mortality in the first 24 hpf and 100% mortality before reaching 48 hpf.

The percentage mortality data at 200 and 400 µg/mL were used to determine the $LC_{50}$ value of the test extract by means of probit analysis. Consequently, the $LC_{50}$ value of the extract was calculated to be 419.84 µg/mL. The logarithmic estimation

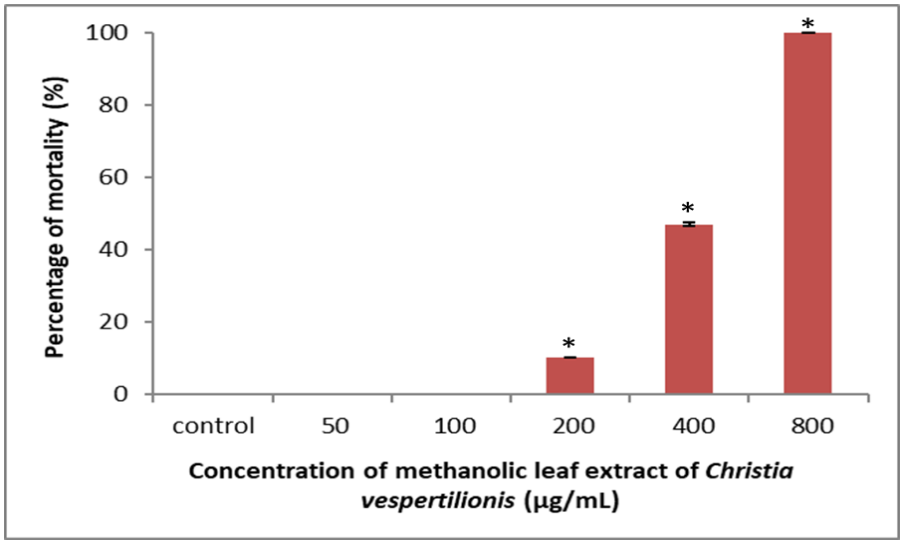



*Figure 1*. Mortality rate of zebrafish embryos exposed to methanolic leaf extract of *Christia vespertilionis*. Values are expressed as mean ± standard deviation of three biological replicates.

*Note*. *Significantly different from the control ($P \leq 0.05$)

of the $LC_{50}$ value is displayed in Figure 2. Generally, higher $LC_{50}$ values imply less test chemical toxicity as greater concentrations are required to elicit 50% mortality in the test organisms (Thiagarajan et al., 2019). Meanwhile, according to the OECD (2013), any toxicants are categorized as 'harmful', 'toxic', and 'highly toxic' if the value of $LC_{50}$ ranges between 10–100 mg/L, 1–10 mg/L, and < 1 mg/L, respectively. Since the $LC_{50}$ value of the extract was higher than the OECD values, it could be concluded, at this stage, that this methanolic extract is non-toxic and safe for consumption, at least for concentrations lower than its $LC_{50}$ value. However, the mortality rate is not the final decisive criterion for the safety of a plant extract. Its effect on the overall development of an organism must also be considered.

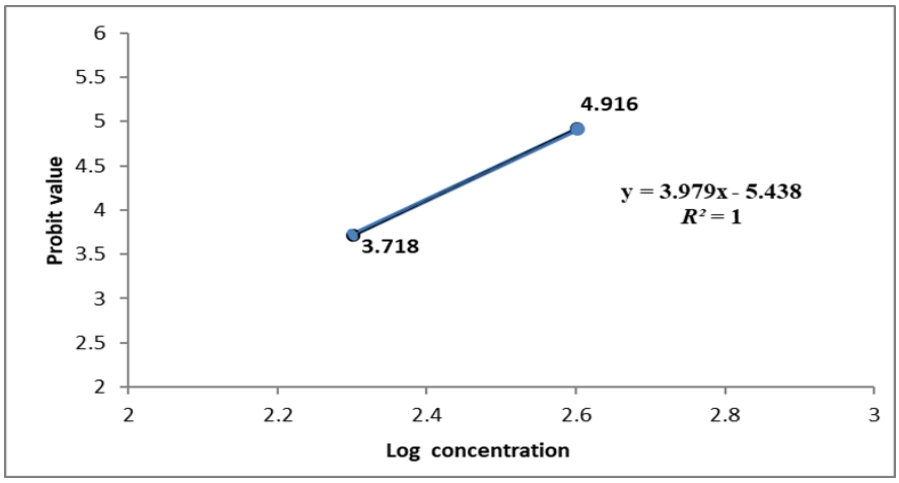



*Figure 2*. Median lethal concentration ($LC_{50}$) value of methanolic leaf extract of *Christia vespertilionis* based on probit analysis

**Effect on Rate of Heartbeat.** The normal heartbeat rate of zebrafish embryos ranges from 120 to 180 beats per minute (bpm) (De Luca et al., 2014). Therefore, the effect of the varying concentrations of the extract on the embryos heartbeat rate was evaluated at 48 hpf; values were expressed as several beats per minute (bpm). The results are shown in Figure 3. There was no significant difference in the mean heartbeat rate between the control group and groups with 50-200 µg/mL concentrations. In contrast, embryos exposed to 400 µg/mL showed a significant decrease in their heartbeat rate with a mean value of 102.067 bpm, compared to the control and the 50-200 µg/mL treatment groups. Meanwhile, no heartbeat was observed in the embryos exposed to the highest 800 µg/mL concentration due to early mortality.

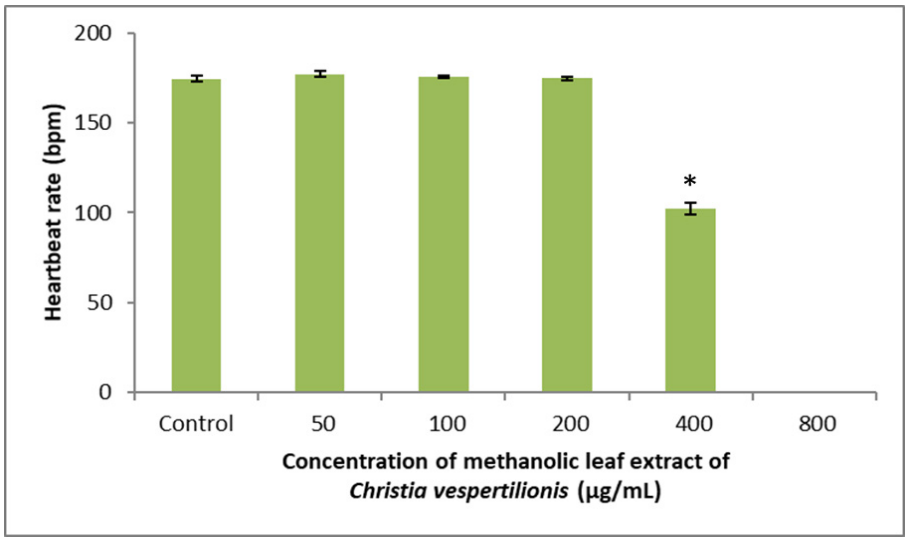



*Figure 3*. Heartbeat rate of zebrafish embryos at 48 hpf exposed to methanolic leaf extract of *Christia vespertilionis*. Values are expressed as mean ± standard deviation of three biological replicates.

*Note*. *Significantly different from the control ($P \leq 0.05$)

**Effect on Hatchability.** During normal embryogenesis of zebrafish, the hatching process is characterized by the breakdown of the chorion, releasing the free-living larvae. This process usually occurs within 48-72 hpf (Thiagarajan et al., 2019). Therefore, the hatchability rate of zebrafish embryos exposed to varying concentrations was evaluated. As presented in Figure 4, the hatchability rate of the exposed embryos was strongly dependent on the concentration of the test extract. At higher concentrations of 400 and 800 µg/mL, no hatching was observed at 72 hpf due to the delayed growth and 100% mortality were recorded even before 48 hpf, respectively. In contrast, 100% hatching was recorded for concentrations of 50, 100, and 200 µg/mL, which was comparable to the control group.

**Effect on Spontaneous Movement of Tail Coiling.** Spontaneous motor activity is an ideal behavioural test for neuronal function. This parameter is commonly used to evaluate the neurotoxic potential of chemical substances (Moser, 2011). The spontaneous movement of tail coiling in zebrafish embryos at 24 hpf was evaluated to determine the motor deficit potentially induced by the varying concentrations of the test extract. The results, as depicted in Figure 5, showed that there was the absence of spontaneous movement of tail coiling at the concentrations of 400 and 800 µg/mL due to their delayed growth and early mortality, respectively. In contrast, no significant changes in the spontaneous movement of tail coiling were observed for the concentrations of 50 to 200 µg/mL compared to the control.

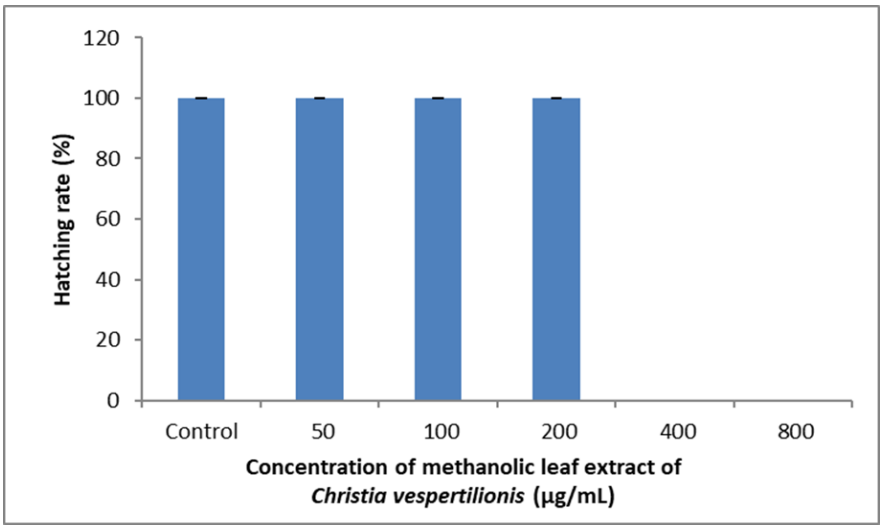



*Figure 4*. Hatching rate of zebrafish embryos exposed to methanolic leaf extract of *Christia vespertilionis*. Values are expressed as mean ± standard deviation of three biological replicates

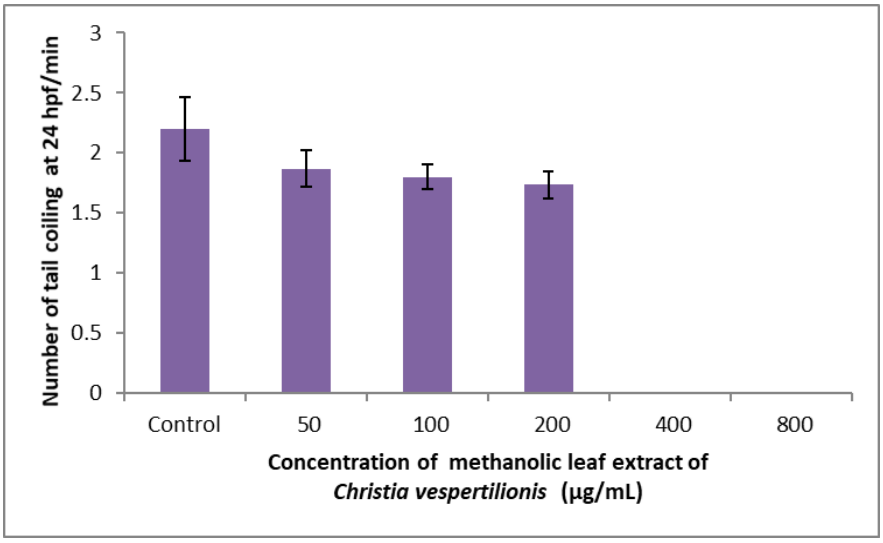



*Figure 5*. Spontaneous tail coiling rate of zebrafish embryos exposed to methanolic leaf extract of *Christia vespertilionis*. Values are expressed as mean ± standard deviation of three biological replicates

## Teratogenic Effects

As shown in Figures 6 and 7, embryos exposed to high concentrations exhibited multiple signs of developmental abnormalities, including delay in development, bent or undetached tail, spinal column curving, pericardial sac oedema, yolk sac oedema, small eyes, abnormal head shape, and uninflated swim bladder. Delayed growth (stage delay) was noted at 24 hpf in the surviving embryos at 400 and 800 µg/mL concentrations (Figure 6), which showed that the embryos were still at 14-somite and 5-somite stages, respectively. In contrast, active embryos with complete detachment of tail from the yolk sac were observed at the concentrations of 50, 100, and 200 µg/mL, comparable with the normal embryos in the control group. After 72 hpf, it was observed that hatched larvae exposed to 200 and 400 µg/mL exhibited severe morphological abnormalities (Figure 7).

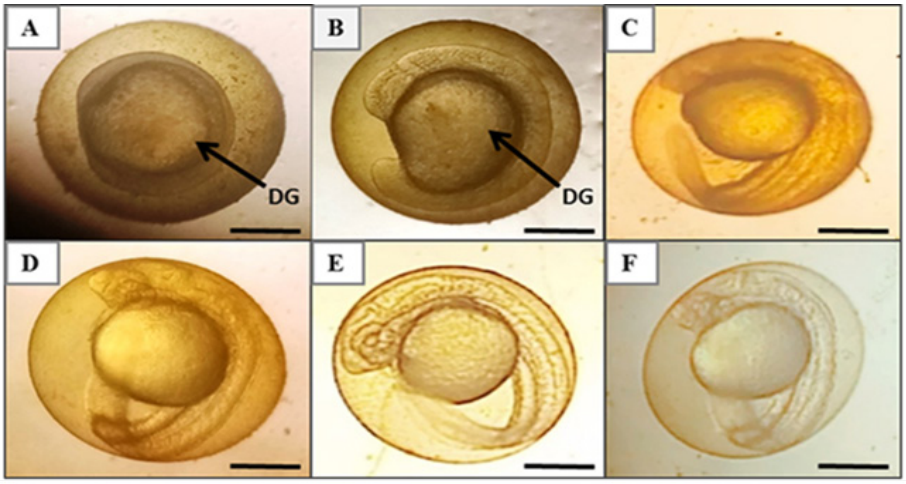
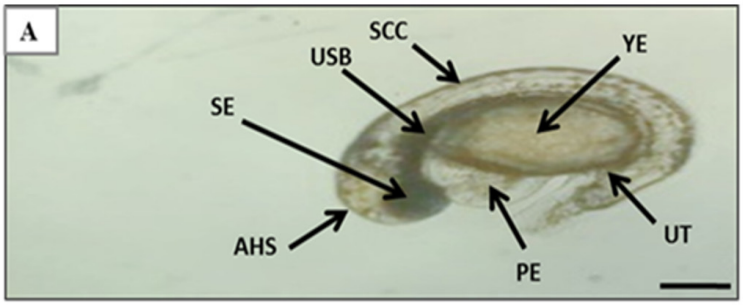



*Figure 6*. Representative optical image of zebrafish embryo exposed to (**A**) 800 µg/mL, (**B**) 400 µg/mL, (**C**) 200 µg/mL, (**D**) 100 µg/mL, (**E**) 50 µg/mL, and (**F**) control at 24 hpf. Malformations are indicated by arrows. DG–delayed growth. Scale bar = 1mm



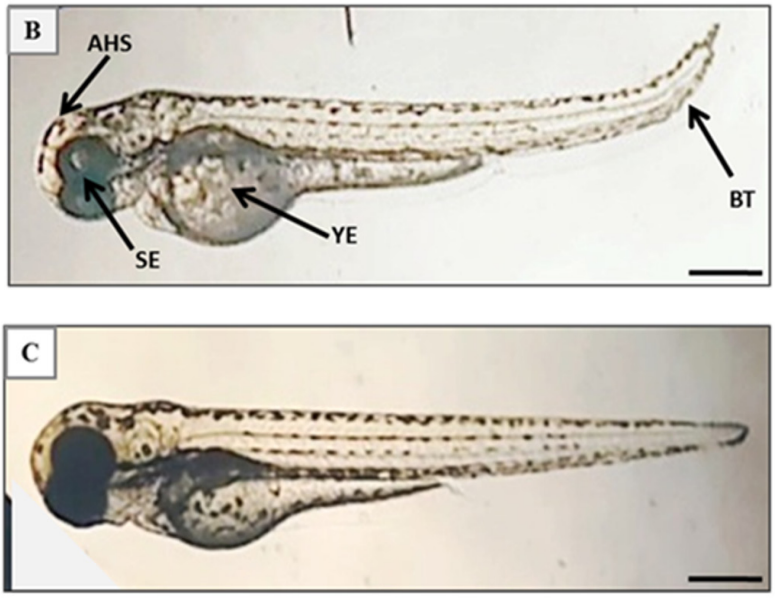



*Figure 7*. Representative optical image of zebrafish larvae after 72 hpf. Malformations were indicated by arrows. Larvae with (**A**) spinal column curving (SCC), uninflated swim bladder (USB), pericardial sac oedema (PE), yolk sac oedema (YE), abnormal head shape (AHS), small eyes (SE), and undetached tail (UT) at 400 µg/mL; (**B**) bent tail (BT), yolk sac oedema (YE), small eyes (SE), and abnormal head shape (AHS) at 200 µg/mL; and (**C**) normal morphology (control). Scale bar = 1mm

## DISCUSSION

According to the OECD guidelines (2013), the leaf extract of *C. vespertilionis* may be considered non-toxic, based strictly on the high $LC_{50}$ value of 419.84 µg/mL. However, the overall embryonic development of the exposed groups indicated that the embryos are affected acutely by a high concentration of the extract. At high concentrations, the extract was lethal and induced a significant decrease in heartbeat and hatchability rates and caused various teratogenic effects on the embryos. The delayed hatching observed at 400 µg/mL indicated growth retardation of the embryos. The delayed hatching may be due to developmental abnormalities in the developing embryos, as evidenced by a severe spinal column curvature in the treated embryos, which limited their ability to break the chorion (Murugesu et al., 2019). The decreased heartbeat rate observed in all surviving embryos at 400 µg/mL suggested that high extract concentrations may cause cardiotoxicity. Consistent with this was the occurrence of oedema in the pericardial sac of the hatched larvae exposed to 400 µg/mL of the extract, which reflected the embryos failed to develop into the normal morphology as observed in the control group. In general, proper function of the heart is crucial for growth and development in the later stages of life since abnormal heart function is known to cause severe developmental effects (Chen et al., 2018).

Thus, the 100% mortality recorded at the highest 800 µg/mL concentration could be related to the test organism's severe cardiac malfunction. Other observed abnormal developments could also have resulted from altered functions of multiple genes during embryonic development. For example, a phenotype with a bent tail malformation has been linked to a disruption of the *cysteine-rich motor neuron1* (*crim1*) gene, specifically affecting vasculature and somites development (Kinna et al., 2008). In the case of spinal column curving, the phenotype could be due to a decrease in collagen synthesis in the spinal column, changes in amino acid composition, or resulting from inhibition or downregulation of *protein tyrosine kinase 7* (*PTK7*) gene, a critical regulator of Wnt signalling (Pamanji et al., 2015).

Despite the prolonged use of plants as a valuable source of pharmacologically active constituents, the phytochemicals it contains could also be potential toxins for humans and animals (Chandra et al., 2012). Similarly, the adverse effects experienced by the embryos upon exposure to high concentrations of the leaf extract of *C. vespertilionis* may be attributable to its phytochemical composition. Phytochemical analysis of the extract revealed it to contain high amounts of polyphenolic constituents, comprising of flavonoids as the major class (mono- and di-hydroxyflavones, *C*-glycosylflavone derivatives, flavone-*C*, *O*-diglycoside, and flavonol-3-*O*-glycosides) and followed by phenolic acids, among other classes of minor constituents (Norazhar et al., 2021).

Previous studies have mostly focused on the beneficial effects of polyphenolic compounds on a broad spectrum of pharmacological properties. However, several studies have reported that high doses of polyphenolic-rich foods can potentially cause adverse effects through pro-oxidative effects (Martin & Appel, 2009). Instead of exhibiting powerful antioxidant activities, high concentrations of polyphenolic compounds can also increase oxidative stress at a cellular level, and thus, increase the risk of diseases. From this perspective, the toxic and teratogenic effects of the leaf extract of *C. vespertilionis* on the embryonic development of zebrafish observed in this study could also be due to the accumulation of high amounts of the flavonoids and phenolic acids constituents in the exposed embryos.

Our findings are similar to the study by Alafiatayo et al. (2019), who reported that high concentrations of methanolic extract of *Curcuma longa*, containing an abundance of the flavonoids catechin, epicatechin, and naringenin, caused mortality and developmental abnormalities in zebrafish embryos. Ismail et al. (2017) also demonstrated that zebrafish embryos exposed to the phenolics-rich aqueous extracts of *Cinnamon zeylanicum* and *Eugenia polyantha* showed a significant toxicity effect after 48 hpf, evidenced by a decrease in survival rate, organ malformations, abnormal heartbeat rates, and delayed hatchability. In another study by Gaitan et al. (1989), the *C*-glycosylflavones-enriched fractions and several purified

*C*-glycosylflavones (glucosylvitexin, glucosylorientin, and vitexin) of pearl millet were shown to inhibit thyroid peroxidase (TPO) *in vitro*. Furthermore, they caused a significant increase in thyroid weight of female Sprague-Dawley rats—these findings demonstrated a strong correlation between high amounts of *C*-glycosylflavones and the genesis of goitre. Doerge and Divi (1995) further proposed that inhibition of TPO, the enzyme responsible for the thyroid hormone production, could be associated with the ability of polyphenolic compounds with free resorcinol (metahydroxyphenol) units to react with the enzyme. Bezerra et al. (2016) also reported that the hydroethanolic extract of *Turnera diffusa*, containing flavone-*C*, *O*-diglycoside as the main constituents, was found to be toxic at high concentrations, specifically at 1000 µg/mL, evidenced by increased cell death of the astrocyte culture after 6 and 24 hours of incubation. Further, Du et al. (2017) reported that intravenous injection of high doses of phenolic acids to male Wistar rats led to an imbalance between oxidant and antioxidant mechanisms, boosting the expression level of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, resulting in damage to microvascular endothelial cells.

There are still relatively few reports on the adverse effects of individual polyphenolic compounds. Therefore, at present, it is not possible to link the observed toxic effects of the individual polyphenolic compounds. Deeper studies on the purified compounds will need to be carried out before any suggestions can be made on their safety levels with regard to human consumption.

## CONCLUSION

The present study revealed that the methanolic leaf extract of *Christia vespertilionis* (green-leafed variety) is toxic to zebrafish embryos at concentrations of 200 µg/mL and above, causing multiple signs of developmental abnormalities. Results of the present study have provided an initial insight into the potentially toxic and teratogenic effects of the extract. Further substantiation of the results and a deeper understanding of the observed effects will require further investigations on other animal or *in vitro* models. Phenolic constituents of the plant are implicated as the cause of the toxicity and teratogenicity of the plant, but the definite proof will also require more detailed studies on the purified constituents. At this stage, based on the results of the present study, extreme caution is advised in using the plant for healthcare purposes at uncontrolled concentrations.

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## REFERENCES

Abd Latip, N., & Abd Mutalib, N. (2019). Synergistic interactions between *Christia vespertilionis* leaves extract and chemotherapy drug cyclophosphamide on WRL-68 cell line. *Asian Journal of Pharmaceutical Research*

*and Development*, *7*(3), 109–113. https://doi.org/10.22270/ajprd.v7i3.488

Alafiatayo, A. A., Lai, K., Syahida, A., Mahmood, M., & Shaharuddin, N. A. (2019). Phytochemical evaluation, embryotoxicity, and teratogenic effects of *Curcuma longa* extract on zebrafish (*Danio rerio*). *Evidence-Based Complementary and Alternative Medicine*, *2019*, 3807207. https://doi.org/10.1155/2019/3807207

Bezerra, A. G., Negri, G., Duarte-Almeida, J. M., Smaili, S. S., & Carlini, E. A. (2016). Phytochemical analysis of hydroethanolic extract of *Turnera diffusa* Willd and evaluation of its effects on astrocyte cell death. *Einstein (São Paulo)*, *14*(1), 56–63. https://doi.org/10.1590/S1679-45082016AO3386

Caballero, M. V., & Candiracci, M. (2018). Zebrafish as screening model for detecting toxicity and drugs efficacy. *Journal of Unexplored Medical Data*, *3*, 4. https://doi.org/10.20517/2572-8180.2017.15

Chandra, S. J, Sandhya, S., Vinod, K. R., David, B., Sudhakar, K., & Chaitanya, R. (2012). Plant toxins-useful and harmful effects. *Hygeia Journal for Drug and Medicines*, *4*(1),79-90.

Chen, L., Xu, M., Gong, Z., Zonyane, S., Xu, S., & Makunga, N. P. (2018). Comparative cardio and developmental toxicity induced by the popular medicinal extract of *Sutherlandia frutescens* (L.) R.Br. detected using a zebrafish Tuebingen embryo model. *BMC Complementary and Alternative Medicine*, *18*(1), 273. https://doi.org/10.1186/s12906-018-2303-9

Clark, T. S., Pandolfo, L. M., Marshall, C. M., Mitra, A. K., & Schech, J. M. (2018). Body condition scoring for adult zebrafish (*Danio rerio*). *Journal of the American Association for Laboratory Animal Science*, *57*(6), 698-702. https://doi.org/10.30802/AALAS-JAALAS-18-000045

Dash, G. K. (2016). An appraisal of *Christia vespertilionis* (L. f.) Bakh. f.: A promising medicinal plant. *International Journal of Pharmacognosy and Phytochemical Research*, *8*(6), 1037-1039.

De Luca, E., Zaccaria, G. M., Hadhoud, M., Rizzo, G., Ponzini, R., Morbiducci, U., & Santoro, M. M. (2014). *ZebraBeat*: A flexible platform for the analysis of the cardiac rate in zebrafish embryos. *Scientific Reports*, *4*, 4898. https://doi.org/10.1038/srep04898

Doerge, D. R., & Divi, R. L. (1995). Porphyrin π-cation and protein radicals in peroxidase catalysis and inhibition by anti-thyroid chemicals. *Xenobiotica*, *25*(7), 761–767. https://doi.org/10.3109/00498259509061891

Du, W. Y., Xiao, Y., Yao, J. J., Hao, Z., & Zhao, Y. B. (2017). Involvement of NADPH oxidase in high-dose phenolic acid-induced pro-oxidant activity on rat mesenteric venules. *Experimental and Therapeutic Medicine*, *13*(1), 17–22. https://doi.org/10.3892/etm.2016.3923

Finney, D. J. (1971). *Probit analysis*. Cambridge University Press.

Gaitan, E., Lindsay, R. H., Reichert, R. D., Ingbar, S. H., Cooksey, R. C., Legan, J., Meydrech, E. F., Hill, J., & Kubota, K. (1989). Antithyroid and goitrogenic effects of millet: Role of *C*-glycosylflavones. *The Journal of Clinical Endocrinology and Metabolism*, *68*(4), 707–714. https://doi.org/10.1210/jcem-68-4-707

Gao, X. P., Feng, F., Zhang, X. Q., Liu, X. X., Wang, Y. B., She, J. X., He, Z. H., & He, M. F. (2014). Toxicity assessment of 7 anticancer compounds in zebrafish. *International Journal of Toxicology*, *33*(2), 98–105. https://doi.org/10.1177/1091581814523142

Ghasemzadeh, A., Jaafar, H. Z., & Rahmat, A. (2015). Phytochemical constituents and biological activities of different extracts of *Strobilanthes crispus* (L.) Bremek leaves grown in different locations of Malaysia. *BMC Complementary and Alternative Medicine*, *15*(1), 422. https://doi.org/10.1186/s12906-015-0873-3

Hofer, D., Schwach, G., Ghaffari Tabrizi-Wizsy, N., Sadjak, A., Sturm, S., Stuppner, H., & Pfragner, R. (2013). *Christia vespertilionis* plant extracts as novel antiproliferative agent against human neuroendocrine tumor cells. *Oncology reports*, *29*(6), 2219–2226. https://doi.org/10.3892/or.2013.2367

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J. C., Koch, R., Rauch, G.-J., White, S., ... Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, *496*(7446), 498-503. http://doi.org/10.1038/nature12111

Hussin, A. H. (2001). Adverse effects of herbs and drug-herbal interactions. *Malaysian Journal of Pharmacy*, *1*(2), 39–44.

Ismail, H. F., Hashim, Z., Soon, W. T., Rahman, N., Zainudin, A. N., & Majid, F. (2017). Comparative study of herbal plants on the phenolic and flavonoid content, antioxidant activities and toxicity on cells and zebrafish embryo. *Journal of Traditional and Complementary Medicine*, *7*(4), 452–465. https://doi.org/10.1016/j.jtcme.2016.12.006

Jayasinghe, C. D., & Jayawardena, U. A. (2019). Toxicity assessment of herbal medicine using zebrafish embryos: A systematic review. *Evidence-Based Complementary and Alternative Medicine*, *2019*, 7272808. https://doi.org/10.1155/2019/7272808

Kinna, G., Kolle, G., Carter, A., Key, B., Lieschke, G. J., Perkins, A., & Little, M. H. (2008). Knockdown of zebrafish *crim1* results in a bent tail phenotype with defects in somite and vascular development. *Mechanisms of Development*, *123*(4), 277–287. http://doi.org/10.1016/j.mod.2006.01.003

Lee, J. J., Saiful Yazan, L., Kassim, N. K., Che Abdullah, C. A., Esa, N., Lim, P. C., & Tan, D. C. (2020). Cytotoxic activity of *Christia vespertilionis* root and leaf extracts and fractions against breast cancer cell lines. *Molecules*, *25*(11), 2610. https://doi.org/10.3390/molecules25112610

Maes, J., Verlooy, L., Buenafe, O. E., de Witte, P. A., Esguerra, C. V., & Crawford, A. D. (2012). Evaluation of 14 organic solvents and carriers for screening applications in zebrafish embryos and larvae. *PLOS One*, *7*(10), e43850. https://doi.org/10.1371/journal.pone.0043850

Martin, K. R., & Appel, C. L. (2009). Polyphenols as dietary supplements: A double-edged sword. *Dove Press*, *2*, 1-12. https://doi.org/10.2147/NDS.S6422

Moser, V. C. (2011). Functional assays for neurotoxicity testing. *Toxicologic Pathology*, *39*(1), 36-45. https://doi.org/10.1177/0192623310385255

Murugesu, S., Perumal, V., Balan, T., Fatinanthan, S., Khatib, A., Arifin, N. J., Shukri, N. S. S. M., Saleh, M. S. M., & Hin, L. W. (2020). The investigation of antioxidant and antidiabetic activities of *Christia vespertilionis* leaves extracts. *South African Journal of Botany*, *133*, 227–235. https://doi.org/10.1016/j.sajb.2020.07.015

Murugesu, S., Uddin, Q., Ibrahim, Z., Fathamah, B., Benchoula, K., Idris, N., & El-Seedi, H. R. (2019). Toxicity study on *Clinacanthus nutans* leaf hexane fraction using *Danio rerio* embryos. *Toxicology Reports*, *6*, 1148–1154. https://doi.org/10.1016/j.toxrep.2019.10.020

Nguyen-Pouplin, J., Tran, H., Tran, H., Phan, T. A., Dolecek, C., Farrar, J., Tran, T. H., Caron, P., Bodo, B., & Grellier, P. (2007). Antimalarial and cytotoxic activities of ethnopharmacologically selected medicinal plants from South Vietnam. *Journal of Ethnopharmacology*, *109*(3), 417–427. https://doi.org/10.1016/j.jep.2006.08.011

Norazhar, A. I., Lee, S. Y., Faudzi, S. M. M., & Shaari, K. (2021). Metabolite profiling of *Christia vespertilionis* leaf metabolome *via* molecular network approach. *Applied Sciences*, *11*(8), 1-29. https://doi.org/10.3390/app11083526

Nurul, S., Hazilawati, H., Mohd, R. S., Mohd, F., Noordin, M. M., & Norhaizan, M. E. (2018). Subacute oral toxicity assesment of ethanol extract of *Mariposa christia vespertilionis* leaves in male Sprague Dawley rats. *Toxicological Research*, *34*(2), 85–95. https://doi.org/10.5487/TR.2018.34.2.085

Organisation for Economic Co-operation and Development. (2013). *Fish Embryo Acute Toxicity (FET) test*. OECD Publishing. https://doi.org/10.1787/9789264203709-en

Pamanji, R., Yashwanth, B., Bethu, M. S., Leelavathi, S., Ravinder, K., & Rao, J. V. (2015). Toxicity effects of profenofos on embryonic and larval development of zebrafish (*Danio rerio*). *Environmental Toxicology and Pharmacology, 39*(2), 887–897. http://doi.org/10.1016/j.etap.2015.02.020

Saint-Amant, L., & Drapeau, P. (1998). Time course of the development of motor behaviors in the zebrafish embryo. *Journal of Neurobiology*, *37*(4), 622–632. http://doi.org/10.1002/(sici)1097-4695(199812)37:4<622::aid-neu10>3.0.co;2-s

Thiagarajan, S. K., Krishnan, K. R., Ei, T., Shafie, N. H., Arapoc, D. J., & Bahari, H. (2019). Evaluation of the effect of aqueous *Momordica charantia* Linn. extract on zebrafish embryo model through acute toxicity assay assessment. *Evidence-Based Complementary and Alternative Medicine*, *2019*, 9152757. https://doi.org/10.1155/2019/9152757

Zakaria, A. (2015, March 13). UPM runs stage two of anti-cancerous red butterfly wing research. *UPM News*. https://upm.edu.my/content/upm_runs_stage_two_of_anti_cancerous_red_butterfly_wing_research-25072

Zhang, C., Willett, C., & Fremgen, T. (2003). Zebrafish: An animal model for toxicological studies. *Current Protocols in Toxicology*, *17*(1), 1-7. https://doi.org/10.1002/0471140856.tx0107s17

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# Investigation of the Best Artificial Propagation Technique for Stingless Bee *Heterotrigona itama* (Hymenoptera: Apidae: Meliponini)

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## ABSTRACT

Meliponiculture (keeping stingless bees) is a practice that is growing rapidly in the tropical and subtropical regions of the world. A limited number of studies regarding the technique would be most accurate in propagating the colonies to increase their numbers. Three different artificial propagation techniques were investigated in Ladang 10, Universiti Putra Malaysia. Three artificial propagation techniques, namely splitting, bridging, and splitting bridging, were conducted for eight consecutive weeks. Honey pot quantity and pollen pot quantity were recorded weekly for eight consecutive weeks. The success of colony division under different artificial propagation techniques and all the parameters taken were observed and recorded weekly. A significant difference ($F = 15.04$, df = 2, $P = <.0001$) was detected in the number of pollen pots between the different artificial propagation techniques, but not for the honey pot quantity ($F = 0.22$, df = 2, $P = 0.8054$). The bridging technique recorded the lowest pollen pot quantity while there was no significant difference in splitting and splitting-bridging techniques. The result showed that the splitting technique obtained new brood cells and queen of *Heterotrigona itama*. The splitting-bridging technique developed new brood cells without a new queen, whereas the bridging technique produced only pollen and honey pots. A matured queen's presence can defeat the artificial propagation technique due to its pheromones function.

*Keywords*: *Heterotrigona itama*, meliponiculture, propagation technique

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## INTRODUCTION

*Heterotrigona itama* is one of the most commercial stingless bees reared in Malaysia (Mustafa et al., 2018). Deforestation reduces the colony of stingless bees and affects their actual role as forest pollinators (Eltz & Bru, 2003). The natural habitat of stingless bees could be destroyed by human activities of cutting down trees or hunting for bee colonies (Villamueva et al., 2005). Cortopassi-Laurino et al. (2006) stated that stingless bees colonies could survive for a long time, typically for more than 50 years. However, the number of swarming times and the queen's lifespan remain unknown. Gradually, new colonies will begin to form as the old colony splits; this is when the new virgin queen leaves for a new house, escorted by a swarm of stingless bee workers (Nunes et al., 2014). A practical way to multiply the stingless bee colony is by constructing an artificial nest, where the process of stingless bee swarming can be performed naturally (Cortopassi-Laurino et al., 2006).

The stingless bee workers will transfer items such as cerumen, resin, and pollen from the old house needed for constructing a new house. Their activities would also aid in providing sufficient nutrients, which were originally transferred from the old house into the new house to develop a new colony (Kwapong et al., 2010). In addition, most stingless bee species have a steady supply of immature virgin queens as protection if the governing queen is killed (Sakagami, 1982). Therefore, the most typical technique for resolving the queen's absence in a split colony is for one of the young genes to develop, fly, and take over the egg-laying duty (Imperatriz-Fonseca & Zucchi, 1995).

It is quite challenging for bee farmers to harvest their nest materials since the stingless bees' nests are often found in tree hollows, dead logs, stems, branches of living trees, and cracks in the wall of houses. Therefore, alternative methods of rearing queen bees and propagating the colony need to be developed without altering the forest biodiversity by mimicking its initial habitat. Resultantly, moving the colony of stingless bees into the artificial hive facilitates the extraction of nest product, simpler to transfer and to propagate (Cortopassi-Laurino et al., 2006).

Splitting or dividing the colonies is another valuable technique. Many people use a crude way to separate their colonies by cutting down whole trees to reach the nests, which results in a lower success rate. However, scientific literature on colony transition and splitting strategies of economically important stingless bee species in Malaysia, such as *H. itama*, is comparatively scarce (Mohd Saufi & Thevan, 2015). This research aims to find the best artificial propagation technique for the stingless bee *H. itama* to expand its population.

## MATERIALS AND METHODS

### Sampling Site

The sampling site was in Ladang 10, Universiti Putra Malaysia, Serdang, Selangor, Malaysia, with a latitude of 2°59'28.7" N longitude 101°42'52.9" E.

Approximately 30 maintained colonies of *H. itama* were present on the farm. The area was surrounded by various fruits trees such as star fruits, mangoes, rambutan, cempedak, and dukong. Mangosteen trees farm was situated about 50 m from the sampling site. Flowering plant, *Antigonon* sp., (Mexican creeper), commonly known as "Air Mata Pengantin" in Malaysia, was also planted near the sampling site.

### Sampling Period and the Hive

This study was conducted from February 2019 to March 2019 for eight consecutive weeks (two months). The hive model (25.5cm x 16.5cm x 16.5cm) was constructed with three compartments of the same size, and one 16 mm diameter entrance hole was located at the lowest compartment.

### Sampling Method

Three different artificial propagation techniques were set up in the experimental plot: i) bridging technique, ii) splitting bridging technique, and iii) splitting technique. Each artificial propagation technique was replicated thrice in the experiment. As a result, the success of colony division (obtained new queen) under three different artificial propagation techniques was observed and recorded. In addition, the total number of honey pots and the number of pollen pots were recorded weekly for eight consecutive weeks in the empty boxes of the bridging technique and both (parent and daughter) colonies in the splitting and splitting-bridging techniques.

**Bridging Technique.** A well-developed and maintained *H. itama* hive's logs in the study site were selected, and the empty medium vertical hive model was placed in front of the log, hooked. A 16 mm hole was drilled in the empty box as an entrance hole to allow foragers to go through it. Stingless bees were only allowed to use the new artificial single way to access their colony in the bridging technique (Klumpp, 2007).

About 10 cm of 16 mm in diameter black poly irrigation pipe was used as a connector between the log's hive and the hive model. Half of an empty 500 ml drinking water bottle was carved and used to cover the log entrance tube before the connecter was attached and secured in place with black duct tape. The roof was provided on top of the hive model. The log and the hive model were kept above the ground using plastic chairs to avoid predators such as ants, termites, toads, and lizards, especially when the nests were situated close to the ground (Kajobe & Roubik, 2006) (Figure 1).

*Figure 1*. Bridging technique. A black poly irrigation pipe with a diameter of 16 mm was used as a connector between the log's hive and the hive model

**Splitting Technique.** The maintained colonies of the *H. itama* bees were obtained by cutting off the log carefully using the Stihl M210 chain saw (Stihl, Germany). The mature stingless bee colony consisted of 9 to14 layers of brood cells (Jaapar et al., 2016). Ten layers of brood cells were transferred into each new hives of which the food sources had been removed (honey and pollen). The layers of the brood cells were placed at the centre of the bottom compartment of the box before closing the lid. A colony was divided into two hive boxes, with one of the hives containing matured brood cells (pupa stage, light brown) and at least two of the virgin queens' cells, while another hive contained young brood cells (larval stage, dark) and a mature queen (Figure 2).

The hive containing the queen was marked with a permanent marker. The hive that contained young brood cells (larval stage, dark brown) was placed at the original position while another hive was placed five meters away from the other box (Quezada-Euán, 2018). The hives were kept above the ground using plastic chairs, so termites were prevented from entering. Tiles (2' x 2') were used as the roof and were placed on top of the hives.

**Splitting Bridging Technique.** The colonies of the *H. itama* were obtained by carefully cutting off the maintained log using the Stihl M210 chain saw (Stihl, Germany). There were two entrance holes sized 16 mm in diameter of each hive. Despite the hive entrance hole, 10 cm length of 16 mm in diameter of black poly irrigation pipe was used as a connector to attach the two hives at the back. The mature stingless bee colony consisted of 9 to 14 layers of brood cells (Jaapar et al., 2016). Furthermore, ten layers of brood cells were transferred into each new hive, of which the food sources were removed (honey and pollen) to avoid attack from natural enemies.

After that, ten layers of the brood cells were placed at the centre of the bottom compartment of the box before closing the lid. A colony was divided into two hive boxes, with one of the hives containing mature brood cells (pupal stage, light brown) and at least two of the virgin queen's

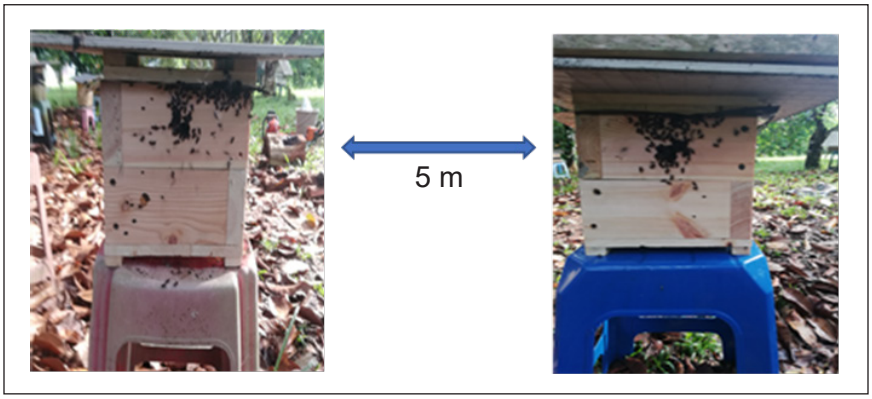


*Figure 2*. Splitting technique. Five-meter distance of each medium hive model

cells. In contrast, the other hive contained young brood cells (larval stage, dark brown) and a queen was placed at their original positions. The hive containing a queen was marked using a permanent marker. The hives were kept above the ground using plastic chairs to deter the predators from entering, and 2 feet × 2 feet tiles were used as a roof on top of the hives (Figure 3).

### Data Analysis

All recorded data were subjected to one-way analysis of variance (ANOVA), and the least significant difference (LSD) mean separation was used at a significant level of 5%. All the analyses were conducted using SAS 9.4 version.


*Figure 3*. Splitting-bridging technique. Two entrance holes sized 16 mm diameter of each hive, 10 cm length of 16 mm in diameter of black poly irrigation pipe was used as a connector to attach the two boxes

## RESULTS AND DISCUSSION

### The Observation and Success Rate of Colony Division under Three Different Artificial Propagation Techniques

**Splitting Technique.** All three colonies used in the splitting technique were successfully divided and obtained a new queen. In the splitting technique, a colony of stingless bees was successfully divided into two colonies. One of the colonies contained a mature queen, while the other contained a new queen that emerged from the virgin queen cell. New queens and brood cells were obtained in the box containing mature brood cells and virgin queen cells (Figure 4). In this study, the emergence of the *H. itama* virgin queen was observed for two weeks after the splitting process. At the same time, the new brood cells were constructed as early as three weeks after the splitting process. The result is consistent with the swarming activities of *Tetragonula laeviceps* reported by Inoue et al. (1984).

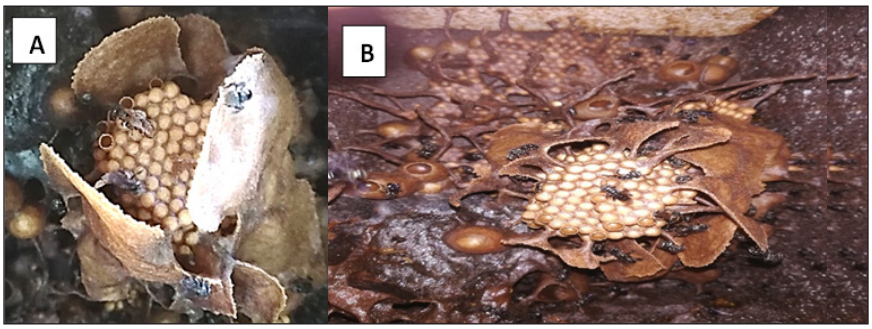


*Figure 4*. Observation after eight weeks in the splitting technique
*Notes*. A colony of stingless bees was successfully divided into two colonies, containing a matured queen (A) and a new queen that emerged from the virgin queen cell (B)

The authors reported that swarming was a rapid process and discovered that a week after the virgin queen's arrival, the daughter colony was independent of the mother's colony. The attractiveness of virgin queens changed after mating. The workers normally produce the brood cells constantly, only if the queen is present.

According to Ahmad Jailani and Abdul Razak (2018), colony splitting is a term used to describe the process of forming two colonies in a specific hive from an established colony to maximise the hive's productivity and separating or splitting the size of bee colonies. When a colony is divided, one of the daughter colonies will have no queen, and most stingless bees' propagation techniques rely on artificially dividing a colony into two daughter colonies (Nunes et al., 2014). However, physically splitting the hive into two halves is considered the quickest and most utilised approach (Dollin, 2001).

Between February and late April is the best period in the Yucatan Peninsula to divide colonies, covering the dry season (Quezada-Euán, 2018). However, it is not advisable to divide colonies during the rainy season, which runs from late May to November, this is due to the increase in the breeding of flies, and there would not be enough food in the field to sustain the establishment of new colonies (González-Acereto et al., 2006). In addition, queen mating may take longer during the rainy season since male production reduces at this time (González-Acereto et al., 2006; Moo-Valle et al., 2000). The dry season, popularly known as the fruit season, is between February and July in Malaysia, but it might change due to weather conditions and the colonies' requirements (Jaapar et al., 2016).

**Splitting-Bridging Technique.** All three colonies used in the splitting-bridging technique were not successfully divided. Although new brood cells were developed in both (parent and daughter) colonies, a new queen was not obtained, and the mature queen controlled the new brood cells. The virgin queen of the daughter colony was unsuccessful to requeen in the splitting-bridging technique, which might be due to the bridge that acted as a tunnel or connector for the mature queen. The bridge or connector provided access to the mature queen to patrol from one hive to another. Regarding the emerging virgin queens, Imperatriz-Fonseca and Zucchi (1995) summarised all three possibilities that could have occurred: i) virgin queen being killed, ii) replaced by the dominant queen, and iii) workers gather to establish a new nest. The queen utilised pheromones to inhibit and monitor their workers (Fletcher & Ross, 1985). Moreover, pheromones indicate the presence of the queen (Nunes et al., 2014). Imperatriz-Fonseca and Zucchi (1995) also reported that the former queen of the colony would be replaced once she became less attractive to the workers. Workers become enraged by the virgin queens' appearance and beauty and begin hunting and murdering them by twisting off their heads and other body parts (Imperatriz-Fonseca & Zucchi, 1995).

**Bridging Technique.** No new queen and brood cells were developed in the empty hives of the bridging techniques. Bridging has become a new and popular method among many native beekeepers for spreading stingless bees (Dollin, 2001). Dollin (2001) also reported that the bridging technique was discovered by Tom Carter and further developed by Klumpp (2007). The bridging or budding technique is very helpful to create a new bud colony in a position where there is no access to remove it from the current parent colony (Heard, 2016).

The bridging technique also requires proper skills to reduce the chances of the parent colony trying to kill the daughter colony queen (Heard, 2016). The stingless bees can also be coaxed into a box using this approach from a natural nest location in a big tree or an inaccessible hole (Dollin, 2001). Several studies have reported that the development of new colonies took about four months in the bridging method (Dollin, 2001; Mythri et al., 2018; Vijayakumar et al., 2013) and could be prolonged until 45 weeks (Heard, 2016).

## Comparison of Honey and Pollen Pot Quantity in Different Artificial Propagation Techniques

As shown in Figure 5, the honey pot quantity was not significantly different between the different artificial propagation techniques ($F$ = 0.22, df = 2, $P$ = 0.8054). In contrast, there was a significant difference ($F$ = 15.04, df = 2, $P$ = <.0001) of pollen pot quantity between the different artificial propagation techniques. Figure 6 shows that the bridging technique recorded the lowest pollen pot quantity while there was no significant difference in splitting and splitting-bridging techniques. The lowest number of pollen pot quantities in the

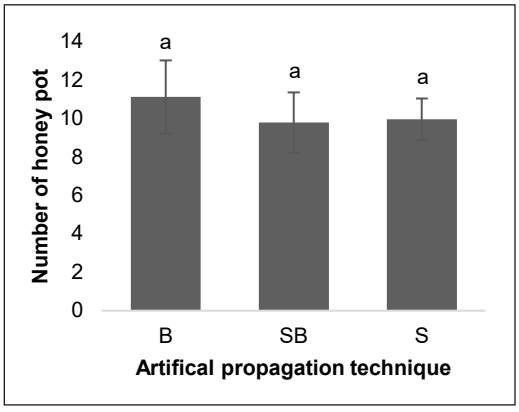


*Figure 5*. Mean numbers of honey pots for *Heterotrigona itama* in different artificial propagation techniques

*Notes*. B = Bridging technique; SB = Splitting-bridging technique; S = Splitting technique. Means with the same letters are not significantly different ($P$>0.05)

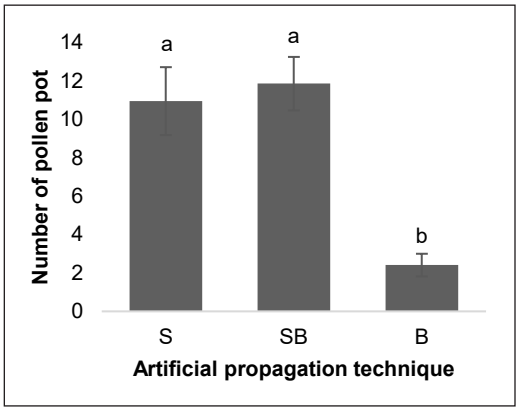


*Figure 6*. Mean numbers of pollen pots for *Heterotrigona itama* in different artificial propagation techniques

*Notes*. B = Bridging technique; SB = Splitting-bridging technique; S = Splitting technique. Means with the same letters are not significantly different ($P$>0.05)

bridging technique indicated slow growth of the colony development.

It might be due to the availability of the existing food storage in the parent colony since there were no brood cells in the empty boxes. The empty box may be accepted as part of their nest and food pots because, in the bridging technique, the parent colony was not removed or transferred from its original location. Pollen was gathered in huge amounts by stingless bees for supplying brood cells or storing pollen pots (Ghazi et al., 2018). Pollen and nectar harvesting efficiency impact a colony's survival, growth, and reproductive success (Maia-Silva, 2014). Thus, pollen is essential for the initial stage of colony development. Most stingless bees get their nitrogen source from pollen, which was gathered in huge amounts by workers for supplying brood cells or storing in colony pollen pots (Ghazi et al., 2018). Roubik and Wheeler (1982) reported that brood production was influenced by the amount of pollen stored in a stingless bee colony.

## CONCLUSION

This study successfully investigated three different artificial propagation techniques for stingless bees, *Heterotrigona Itama*, with the splitting technique being the only successful one. The bridging technique took a very long time (>4 months) for a colony to propagate and needed proper skills to reduce the chances of the parent colony trying to kill the virgin queen of the daughter colony. New brood cells were developed in the splitting-bridging technique but no new queen. The distance between two colonies once divided influenced the success of colony division. The presence of a mature queen can defeat the artificial propagation technique due to its pheromones function.

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## REFERENCES

Ahmad Jailani, N. M. A., & Abdul Razak, M. (2018). Stingless bee rearing and colony splitting. *Pertanika Journal of Scholarly Research Reviews*, *4*(3), 62-69.

Cortopassi-Laurino, M., Imperatriz-Fonseca, V. L., Roubik, D. W., Dollin, A., Heard, T., Aguilar, I., & Nogueira-Neto, P. (2006). Global meliponiculture: Challenges and opportunities. *Apidologie*, *37*(2), 275-292. https://doi.org/10.1051/apido:2006027

Dollin, A. (2001). *Natural hive duplication: An alternative method of propagating Australian stingless bees*. http://www.aussiebee.com.au/aussiebeeonline003.pdf

Eltz, T., & Bru, C. A. (2003). Nesting and nest trees of stingless bees (Apidae: Meliponini) in lowland dipterocarp forests in Sabah, Malaysia, with implications for forest management. *Forest Ecology and Management*, *172*(2-3), 301–313. https://doi.org/10.1016/s0378-1127(01)00792-7

Fletcher, D. J. C., & Ross, K. G. (1985). Regulation of reproduction in eusocial Hymenoptera. *Annual Review of Entomology*, *30*, 319–343. https://doi.org/10.1146/annurev.en.30.010185.001535

Ghazi, R., Zulqurnain, N. S., & Azmi, W. A. (2018). Melittopalynological studies of stingless bees from the east coast of peninsular Malaysia. In P. Vit, S. Pedro, & D. Roubik (Eds.), *Pot-pollen in stingless bee melittology* (pp. 77-88). Springer. https://doi.org/10.1007/978-3-319-61839-5_6

González-Acereto, J., Quezada-Euan, J. J., & Medina-Medina, L. (2006). New perspectives for stingless beekeeping in the Yucatan: Results of an integral program to rescue and promote the activity. *Journal of Apicultural Research*, *45*(4), 234-239. https://doi.org/10.1080/00218839.2006.11101356

Heard, T. (2016). *The Australian native bee book: Keeping stingless bee hives for pets, pollination and sugarbag honey*. Sugarbag Bees.

Imperatriz-Fonseca, V. L., & Zucchi, R. (1995). Virgin queens in stingless bee (Apidae, Meliponinae) colonies: A review. *Apidologie*, *26*(3), 231-244. https://doi.org/10.1051/apido:19950305

Inoue, T., Sakagami, S. F., Salmah, S., & Yamane, S. (1984). The process of colony multiplication un the Sumatran stingless bee *Trigona* (*Tetragonula*) *laeviceps*. *Biotropica*, *16*(2), 100-111. https://doi.org/10.2307/2387841

Jaapar, M. F., Halim, M., Mispan, M. R., Jajuli, R., Saranum, M. M., Zainuddin, M. Y., & Ghani, I. A. (2016). The diversity and abundance of stingless bees (Hymenoptera: Meliponini) in peninsular Malaysia. *Advances in Environmental Biology*, *10*(9), 1-7.

Kajobe, R., & Roubik, D. W. (2006). Honey-making bee colony abundance and predation by apes and humans in a Ugandan Forest Reserve. *Biotropica*, *38*(2), 210-218. https://doi.org/10.1111/j.1744-7429.2006.00126.x

Klumpp, J. (2007). *Australian stingless bees: A guide to sugarbag beekeeping*. Earthling Enterprises.

Kwapong, P., Aidoo, K., Combey, R., & Karikari, A. (2010). *Stingless bees: Importance, management and utilisation: A training manual for stingless beekeeping*. Unimax Macmillan.

Maia-Silva, C., Imperatriz-Fonseca, V. L., Silva, C. I., & Hrncir, M. (2014). Environmental windows for foraging activity in stingless bees, *Melipona subnitida* Ducke and *Melipona quadrifasciata* Lepeletier (Hymenoptera: Apidae: Meliponini). *Sociobiology*, *61*(4), 378-385.

Mohd Saufi, N. F., & Thevan, K. (2015). Characterization of nest structure and foraging activity of stingless bee, *Geniotrigona thoracica* Smith (Hymenoptera: Apidae; Meliponini). *Jurnal Teknologi*, *77*(33). https://doi.org/10.11113/Jt.V77.7007

Moo-Valle, H., Quezada-Euán, J. J. G., Navarro, J., & Rodriguez-Carvajal, L. A. (2000). Patterns of intranidal temperature fluctuation for *Melipona beecheii* colonies in natural nesting cavities. *Journal of Apicultural Research*, *39*(1-2), 3-7. https://doi.org/10.1080/00218839.2000.11101015

Mustafa, M. Z., Yaacob, N. S., & Sulaiman, S. A. (2018). Reinventing the honey industry: Opportunities of the stingless bee. *Malaysian Journal of Medical Sciences*, *25*(4), 1-5. https://doi.org/10.21315/mjms2018.25.4.1

Mythri, P. G., Kencharaddi, R. N., & Hanumantharaya, L. (2018). Colony division techniques for stingless bee, *Tetragonula iridipennis* Smith. *International Journal of Pure and Applied Bioscience*, *6*(6), 1258-1263. https://doi.org/10.18782/2320-7051.7042

Nunes, T. M., Mateus, S., Favaris, A. P., Amaral, M. F., von Zuben, L. G., Clososki, G. C., Bento, J. M., Oldroyd, B. P., Silva, R., Zucchi, R., Silva, D. B., & Lopes, N. P. (2014). Queen signals in a stingless bee: Suppression of worker ovary activation and spatial distribution of active compounds. *Scientific Reports*, *4*, 7449. https://doi.org/10.1038/srep07449

Quezada-Euán, J. J. G. (2018). Managing and preserving stingless bees. In H. Moo-Valle (Ed.), *Stingless bees of Mexico* (pp. 193-242). Springer. https://doi.org/10.1007/978-3-319-77785-6_8

Roubik, D. W., & Wheeler, Q. (1982). Flightless beetles and stingless bees: Phoresy of scotocryptine beetles on their meliponine hosts. *Journal of the Kansas Entomological Society*, *55*(1), 125–135.

Sakagami, S. F. (1982). Stingless bees. In H. R. Herman (Ed.), *Social insects III* (pp. 361- 423). Academic Press.

Vijayakumar, K., Muthuraman, M., & Jayaraj, R. (2013). Propagating *Trigona iridipennis* colonies (Apidae: Meliponini) by reduction method. *Academic Journal of Bioscience*, *1*(1), 1-3.

Villamueva-G, R., Roubik, D. W., & Colli-Ucán, W. (2005). Extinction of *Melipona beecheii* and traditional beekeeping in the Yucatán peninsula. *Bee World*, *86*(2), 35-41. https://doi.org/10.1080/0005772X.2005.11099651

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# Development of Polyculture Engineering Technology on Milkfish and Mud Crab Farming

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## ABSTRACT

The current study aimed to evaluate the role of polyculture engineering technology on milkfish and mud crab farming and observe the growth and survivability of different milkfish and mud crab combinations. The study used milkfish, which was received artificial feed containing 35 % protein content enriched with vitamin E (3%/biomass/day). The study used completely randomized design with 4 treatments ($n$ = 3): T1 = 5 milkfish + 5 mud crab, T2 = 10 milkfish + 5 mud crab, T3 = 5 milkfish + 10 mud crab, T4 = 10 milkfish + 10 mud crab. The following parameters were measured: absolute weight growth, survival rate, feed conversion rate (FCR), and water quality. The difference in the density of milkfish and mud crabs significantly affected ($p < 0.05$) the growth and survivability of milkfish and mud crabs. The polyculture cultivation system exhibited a significant increase in absolute weight growth of milkfish and mud crabs, which is the highest increase found in T4 treatment (187.85 g ± 0.9 g and 60.65 g ± 0.95 g, respectively). Meanwhile, the survival rate of milkfish and mud crab was 95% ± 0.3% and 95% ± 2.3%, respectively, followed by a lower FCR at T4 (1.54 ± 0.10). The water quality remained good for fish and mud crabs to survive. Milkfish and mud crab polyculture greatly affect the abundance of phytoplankton, demonstrating good community structure.

*Keywords*: Growth, milkfish, mud crabs, polyculture, survivability

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## INTRODUCTION

Nowadays, the problem in cultivating milkfish and mud crab is high mortality (80 % to 95 %). It is due to bacterial attack through nutritional intake of feed such as fish waste and the low quality of environmental water (Samidjan & Rachmawati, 2016). Recently, aquaculture production has increased dramatically, as evidenced by a model structure of biomass production that could boost biomass production by modifying harvesting techniques (Suhartono & Istiyanto, 2014). Furthermore, because of its high economic value, which can be sold abroad, the mangrove crab is one of the most crucial cultivated species globally, particularly in Asia (Samidjan & Rachmawati, 2016).

According to Samidjan and Rachmawati (2016), polyculture black tiger shrimp, fish, and seaweed aquaculture produced poor results. Similarly, several other studies in polyculture cultivation of milkfish, black tiger shrimp, vannamei shrimp, seaweed, and mud crab have led to the development of fisheries in Indonesia. The use of aquacultural technology and the expansion of mud crab farming (*Scylla paramamosain*). It used battery plastic models in ponds to promote the export trade fishery to enhance productiveness. Furthermore, Samidjan and Rachmawati (2016) investigated the innovation of polyculture technology through biofilter systems and different stocking densities of milkfish and white shrimp in water quality improvement, leading to a higher performance of milkfish in the feasibility of white shrimp and milkfish life. Therefore, the current study sought to evaluate the role of technology engineering in polyculture milkfish and mud crab farming and observe the growth and survivability of different milkfish and mud crab combinations.

## MATERIALS AND METHODS

### Preparation of Milkfish and Mud Crab

This study used milkfish [*Chanos chanos* (Forsskal, 1775)] with 5 cm ± 0.025 cm in length and mud crab [*Scylla paramamosain* (Estampador, 1949)] with 4.85 cm ± 1.02 cm in length. The number of fish seeds used was five individuals of milkfish (MF)/$m^2$ to ten individuals of milkfish (MF)/$m^2$ and between five individuals of mud crab (MC)/$m^2$ to 1,200 $m^2$ and ten individuals of mud crab/$m^2$ to 1,200 $m^2$ pond culture. The fish were then received artificial feed containing 35 % protein supplemented with vitamin E (0.9 g/kg feed and 3% feed/biomass/day) (Table 1). Pure protein and vitamin E was purchased from Toko Kimia Indrasari and Sarika Majapahit Pharmacy, Semarang, respectively.

Table 1
*Test feed formulation*

| Raw material of feed composition | (g/100g) |
|---|---|
| Vitamin E | 0.9 |
| Fish flour | 34.3 |
| Soy flour | 33.3 |
| Corn starch | 9.4 |
| Bran flour | 8.1 |
| Dextrin | 9.2 |
| Fish oil | 1.31 |
| Corn oil | 1.31 |
| Mineral vitamin | 1.1 |
| Carboxymethyl cellulose (CMC) | 1.1 |
| Total | 100 |
| Proximate analysis | |
| Protein (%) | 35 |
| Lipid (%) | 11.5 |
| Nitrogen free extract (NFE) (%) | 33.75 |
| Energy (cal/g) | 300.05 |
| Ratio of energy/protein (E/P) (kcal/g) | 8.57 |

## Experimental Design

The current study used a completely randomized design with 4 treatments ($n = 3$): T1 = 5 MF + 5 MC (feeding five individuals of milkfish/$m^2$ and five individuals of mud crab/$m^2$), T2 = 10 MF + 5 MC (feeding ten individuals of milkfish/$m^2$ and five individuals of mud crab/$m^2$), T3 = 5 MF + 10 MC (feeding five individuals of milkfish/$m^2$ and ten individuals of mud crab/$m^2$), T4 = 10 MF + 10 MC (feeding ten individuals/$m^2$ milkfish and ten individuals of mud crab/$m^2$).

## Plankton Abundance

**Diversity Index.** Diversity index was calculated using Shannon Wiener's diversity index (Spellerberg & Fedor, 2003):

$$H' = -\sum_{i=1}^{s} p_i ln p_i \quad (1)$$

where:
$H'$ : Shannon Wiener's diversity index
$p_i$ : Individuals/Total individual (ni/N)
ln : The natural logarithm
S : Number of species

**Uniformity Index.** The following formula below was used to calculate the uniformity index (Ulfah et al., 2019):

$$E = \frac{H'}{Hmax} \quad (2)$$

where:
*H'* : Shannon-Wiener diversity index
$H_{max}$: Maximum *H'* (ln S)
S : Number of species

**Dominance Index.** The following formula below was used to calculate the dominance index (Samidjan et al., 2020):

$$D = \frac{\sum_{i=1} ni(ni-1)}{N(N-1)} \quad (3)$$

where:
D : Dominance index
$n_i$ : Number of individuals
N : Total number of individuals

## Growth Parameter

**Absolute Growth Rates.** The absolute growth rate was determined using the following formula (Samidjan et al., 2020):

$$W = W_t - W_0$$

where:
W : Absolute growth rate
$W_t$ : Final weight (g)
$W_0$ : Initial weight (g)

**Feed Conversion Ratio (FCR).** The feed conversion ratio was measured using the following formula (Samidjan et al., 2020):

$$FCR = \frac{F}{(W_t + d) - W_0}$$

where:
FCR: Food conversion ratio
F : Food consumed (g)
$W_0$ : Initial weight (g)
Wt : Final weight (g)

**Survival Rate.** The following formula was used to calculate the survival rate of animals (Samidjan et al., 2020):

$$SR = \frac{N_t}{N_0} \times 100\%$$

where:
SR : Survival rate
$N_0$ : Initial number of animals
$N_t$ : Final number of animals

## Water Quality Parameter

Water pH and dissolved oxygen were measured using Jenway 3510 standard digital pH meter (Jenway, United Kingdom) and Jenway 970 dissolved oxygen meter (Jenway, United Kingdom). In addition, the ammonia level, temperature, and salinity were measured using a HI-8633 portable conductivity meter (Hanna Instruments Inc., USA).

## Statistical Analysis

Data were included absolute growth of milkfish (g), absolute growth of mud crab (g), the survival rate of milkfish (%), the survival rate of mud crab (%), and FCR of milkfish and mud crab. Data were expressed as mean ± standard deviation (SD) and analyzed using analysis of variance

(ANOVA), and Duncan's multiple range test (DMRT) with $p < 0.01$ was used as statistical significance.

## RESULTS AND DISCUSSION

The highest absolute weight growth of milkfish and mud crabs was detected in T4 treatment, 187.85 g ± 0.9 g and 60.65 g ± 0.95 g, respectively. In addition, the survival rate of milkfish was 95% ± 0.3% and 95% ± 2.3% for mud crab, while lower feed conversion (FCR) of T4 was 1.54 ± 0.10 (Table 2).

Table 2

*Absolute growth of milkfish and mud crab*

| Parameter | Treatments in polyculture | | | |
|---|---|---|---|---|
| | T1 (5 MF + 5 MC) | T2 (10 MF + 5 MC) | T3 (5 MF + 10 MC) | T4 (10 MF + 10 MC) |
| Absolute growth of milkfish (g) | $180.18 \pm 3.14^{b}$ | $184.27 \pm 0.49^{ab}$ | $185.18 \pm 0.61^{a}$ | $187.85 \pm 0.9^{a}$ |
| Absolute growth of mud crab (g) | $47.85 \pm 0.95^{c}$ | $54.45 \pm 0.62^{b}$ | $58.76 \pm 0.75^{a}$ | $60.65 \pm 0.95^{a}$ |
| Survival rate milkfish (%) | $81.67 \pm 1.81^{b}$ | $85.40 \pm 4.15^{b}$ | $94.07 \pm 2.16^{a}$ | $95 \pm 0.3^{a}$ |
| Survival rate of mud crab (%) | $78.13 \pm 1.10^{b}$ | $81.0 \pm 3.12^{b}$ | $93.43 \pm 1.0^{a}$ | $95 \pm 2.3^{a}$ |
| FCR of milkfish and mud crab | $3.45 \pm 0.43^{a}$ | $2.89 \pm 0.48^{a}$ | $2.09 \pm 0.33^{b}$ | $1.54 \pm 0.10^{b}$ |

*Note.*
T1 = 5 MF + 5 MC (feeding 5 milkfish/$m^2$ and 5 mud crab/$m^2$)
T2 = 10 MF + 5 MC (feeding 10 milkfish/$m^2$ and 5 mud crab/$m^2$)
T3 = 5 MF + 10 MC (feeding 5 milkfish/$m^2$ and 10 mud crab/$m^2$)
T4 = 10 MF + 10 MC (feeding 10 milkfish/$m^2$ and 10 mud crab/$m^2$)

MF = Milkfish; MC = Mud crab; Data were expressed as values ± SD and analyzed using analysis of variance ($p<0.01$). Different superscript letters in the same rows indicated highly significant differences between group treatments ($p<0.01$)

**Absolute Weight Growth of Milkfish**

T4 polyculture exhibited the milkfish's highest absolute weight growth (Table 2). This feeding treatment enhanced the absolute weight growth of milkfish (187.85 g ± 0.9 g), which is higher than the T1, T2, and T3 group ($p < 0.01$). The used polyculture milkfish and mud crab enhance growth and improve absolute growth.

The artificial feeding of milkfish containing 35% protein enriched with vitamin E increased the absolute weight growth of milkfish from 179.5 g to 185.25 g (Agbayani, 2001; Gaillard, 2010; Martan, 2008; Primavera, 2006). Changes in the number of cells that make up human tissue and morphologically changing observable growth are signs of physical growth. When the energy requirements for metabolism and body growth have been met, growth will occur (Araújo-Silva et al., 2014; Chopin, 2013; Davis, 2011; Martan, 2008; Samidjan & Rachmawati, 2018; Siskey & Baldwin, 2011; Yuan et al., 2010). It had also happened when the quantities of feed consumed were more than what was required for body growth, and the fish used it as an energy source (Lall, 2000).

**Absolute Growth of Mud Crabs.** The polyculture technique of rearing milkfish and mud crabs in the same pond with each plot of 100 $m^2$ had a strong influence on absolute mud crab weight ($p < 0.1$) (Table 1). T4 group had the highest absolute weight of mud crab (60.65 g ± 0.025 g).

Furthermore, a highly significant difference was found in mud crab absolute weight growth ($p < 0.01$). It was related to the simultaneous maintenance of mud crabs and milkfish, which can grow well and thus have an excellent synergistic relationship. Adding vitamin E-enriched artificial feed to the diet resulted in optimal growth because it serves as an antioxidant to reduce highly unsaturated fatty acid (HUFA) oxidation. As a result, HUFA availability in the feed can be conserved (Agbayani, 2001; Gaillard, 2010; Xie et al., 2011), and HUFA oxidation in the cell membrane or intercellular free radicals can be eliminated. Indirect feed contributes to the growth and survival rate of metabolism in addition to vitamin E enrichment (Agbayani, 2001; Davis, 2011; Gaillard, 2010; Yang & Fitzsimons, 2002).

Mud crabs have a remarkable capacity to absorb vitamin E, allowing them to gain weight. Vitamin E could prevent oxidative damage, such as carotene degradation in the gut, by performing as an antioxidant (Asadujjaman et al., 2015; Ghosh et al., 2011; Ihsan, 2012; Malleo, 2011; Miroslav et al., 2011; Monwar et al., 2017; Nunes et al., 2003; Sun & Boyd, 2013; Venugopal et al., 2012). Vitamin E has been shown to reduce cell membrane damage, allowing metabolic processes to run more smoothly and nutrients to enter cells appropriately (Agbayani, 2001; Gaillard, 2010; Solomon & Ezigbo, 2010). Herbivorous fish are expected to possess more vitamin E than carnivorous fish (Laxmappa & Khrisna, 2015). The feed requirement for red sea

bream was 442 mg/kg of feed (Ali et al., 2009).

An increase in body size is referred to as growth. The rate of absolute weight growth on the mud crab began with the rate of carapace (shell) width and length growth (Agbayani, 2001; Gaillard, 2010). Because the body cannot grow linearly, absolute weight growth may be critical for mud crabs. The mud crab can grow when the old shell is removed and replaced with a new and larger shell. The process of this change was called the molting process. Molting crabs have been discontinued due to their hard and inelastic shells, as the molting process softens the shell (Agbayani, 2001; Gaillard, 2010).

**Survival Rate of Milkfish and Mud Crab.** The maximum survival rate of fish maintained at T4 treatment was 95% ± 2.3% (Table 2). The milkfish had a good survival rate due to the high-water quality in the maintained fish polyculture system. It was supported by Barman et al. (2012), who mentioned that adequate water quality in polyculture might boost the survival rate up to 80%–90%. Therefore, water quality in fish farming could affect survival, proliferation, and growth. T4 treatment had the highest mud crab survival rate (95% ± 2.3 %) (Table 2).

**Food Conversion Ratio (FCR).** In the polyculture milkfish and mud crab farming system, the feed conversion ratio is crucial because it decides whether the feed can improve the growth of fish and mud crabs still growing well (Davis, 2011). The feed conversion values can also determine how much the feed broadened the mud crab or kept fish body. A lower feed conversion rate (FCR) at T4 resulted in a higher absolute weight of high growth, implying a more efficient feed. Table 2 shows that artificial feed with a reduced FCR value for T4 given to the polyculture system effectively increased mud crab growth.

The feed conversion ratio indicates how many grams of feed are required to create one gram of milkfish bodyweight. Feed efficiency is obtained by calculating the FCR as the value consumed per fish weight unit. A good quality feed has a reduced conversion ratio (FCR), which improves the feed's performance and improves absolute growth (Gaillard, 2010). It was determined as a feed conversion index based on total feed used for growth, with lower values indicating higher feed conversion. It was efficient when the feed conversion value was less than 3. Vitamin E supplements in the diet may potent antioxidants, assisting in preserving vitamins (Agbayani, 2001; Gaillard & Juliette, 2010). The proper nutrients in the feed have an impact on the feed utilization rate because it will help the milkfish and mud crab grow faster in polyculture (Ali et al., 2009; De-shang & Shuang-lin, 2000; Jamerlan et al., 2014; Jaspe et al., 2011; Laxmappa & Khrisna, 2015; Solomon & Ezigbo, 2010).

**The Abundance of Phytoplankton.** Bacillariophyceae (8 genera), Chlorophyceae (1 genus), Cyanophyceae (1 genus), and

Dinophyceae (1 genus) were detected in aquaculture systems polyculture of milkfish and mud crab (Tables 3 and 4). Furthermore, the study found 118.75 individu/L of phytoplankton species, which is higher than milkfish and mud crab. According to Dolgov and Prokopchuk (2018), the number of phytoplankton is higher than in the polyculture system of milkfish and mud crab using biofloc in ponds. The constant availability of nutritional components through the feed is responsible for the high percentage of phytoplankton. The increase in genus and individuals is related to feeding and fertilizer (Napiórkowska-Krzebietke, 2017).

Table 3

*Plankton genus observed during the study*

| Treatment | Phytoplankton genera |
|---|---|
| T1 (5 MF + 5 MC) | *Ceratium*, *Coscinodiscus*, *Bacteriastrum*, *Chaetoceros*, *Geotrichia*, *Navicula*, *Odontella*, *Oscillatoria*, *Pleurosigma* |
| T2 (10 MF + 5 MC) | *Chaetoceros*, *Ceratium*, *Coscinodiscus*, *Bacteriastrum*, *Geotrichia*, *Navicula*, *Odontella*, *Oscillatoria*, *Thalassionema* |
| T3 (5 MF + 10 MC | *Coscinodiscus*, *Geotrichia*, *Navicula*, *Bacteriastrum*, *Chaetoceros*, *Ceratium*, *Pleurosigma*, *Thalassionema* |
| T4 (10 MF + 10 MC) | *Bacteriastrum*, *Oscillatoria*, *Pleurosigma*, *Thalassionema*, *Chaetoceros*, *Ceratium*, *Coscinodiscus*, *Geotrichia*, *Navicula*, *Odontella* |

*Note*. MF = Milkfish; MC = Mud crab

Table 4

*The diversity index (H'), uniformity (E) and dominance (D) phytoplankton*

| Treatments | Number of individuals (individu/L) | Index | | |
|---|---|---|---|---|
| | | Diversity (*H'*) | Uniformity (E) | Dominance (D) |
| T1 (5 MF + 5 MC) | 112 | 1.093 | 0.765 | 0.725 |
| T2 (10 MF + 5 MC) | 115 | 1.072 | 0.753 | 0.606 |
| T3 (5 MF + 10 MC) | 119 | 1.804 | 0.785 | 0.595 |
| T4 (10 MF + 10 MC) | 129 | 1.907 | 0.895 | 0.578 |
| Mean | 118.75 | 1.469 | 0.7995 | 0.626 |

*Note*. MF = Milkfish; MC = Mud crab

This study found that an increase in phytoplankton abundance caused by several factors, such as planktonic genera during the dry season, could enhance the abundance of some genera. During the rainy season, it can raise the phytoplankton abundance. Temperature, nutrient concentration, predation of milkfish and mud crab, pH, disease, weather, phytoplankton, light, competence between species, and algae toxins influence the phytoplankton abundance (Sun & Boyd, 2013). The low abundance of phytoplankton grows very densely simultaneously (Kwon et al., 2018). The addition of feed significantly affected the cultivation of milkfish and mud crab polyculture systems within ponds ($p < 0.05$).

The milkfish and mud crab have an impact on phytoplankton abundance and community structure. Phytoplankton as substitute feed resulted in decreased feed intake without declining feed ratio (Tan et al., 2016). Therefore, FCR can predict the feed required for phytoplankton and seaweed maintenance. Similarly, adding natural food and other feed will reduce FCR values close to or equal 1 (Samidjan et al., 2019). According to Table 4, the diversity index (*H'*) in T1, T2, T3, and T4 treatments were 1.093, 1.072, 1.804, and 1.907, respectively. The average diversity value was 1.469 (*H*'> 1). The plankton conditions in pond waters are shown to be relatively good. This result indicates that the community's condition (plankton, milkfish, and mud crab) has remained generally steady as the pond's environment changes. If *H'* is less than 1, the biota community is unstable (Basmi, 2000). The biota community is classified as moderately stable if *H'* is between 1–3 and as stable if *H'* is more than 3.

Table 4 shows that the uniformity index of T1, T2, T3, and T4 treatment was 0.765, 0.753, 0.785, and 0.895, respectively. The average uniformity index was 0.7995, indicating that the number of individuals in each genus is relatively similar. If E is greater than 0.75, the uniformity value is high, while the value of E is less than 0.75, the uniformity value is low (Table 4). The dominance index of T1, T2, T3, and T4 treatment was 0.725, 0.606, 0.595, and 0.578, respectively. The average dominance index was 0.626, suggesting that no phytoplankton genus dominates the other genus. According to Ali et al. (2009), the dominance index ranges from 0 to 1, with zero indicating no genus dominating the other genus in the biota community structure.

**Water Quality.** Water quality maintenance for milkfish and mud crab polyculture media was crucial for cultivation success. Table 5 revealed that the dissolved oxygen (4.87 mg/L to 6.25 mg/L), temperature (27.5 °C to 31.25 °C), salinity (22 g/L to 28.5 g/L), pH (7.5 to 8.5), and ammonia (0.02 mg/L to 0.256 mg/L) could support fish life and mangrove crabs cultivated in polyculture.

Table 5
*Water quality parameters in polyculture system of milkfish and mud crabs*

| Parameters | Results | Reference (Sun & Boyd, 2013) |
|---|---|---|
| Dissolve oxygen (mg/L) | 4.87 to 6.25 | 4 mg · $L^{-1}$ |
| Temperature (°C) | 27.5 to 31.25 | 26.5 to 35 °C |
| Salinity (g/L) | 22 to 28.5 | 15 to 30 ppt |
| pH | 7.5 to 8.5 | 7.5 to 8.7 |
| Ammonia (mg/L) | 0.02 to 0.256 | < 1 mg · $L^{-1}$ |

## CONCLUSION

The study revealed that the difference in the density of milkfish and mud crabs exhibited a significant effect ($p < 0.05$) on the growth and survivability of milkfish and mud crabs. The polyculture cultivation system showed a significant increase in absolute weight growth of milkfish and mud crabs, which is the highest increase found in T4 treatment (187.85 g ± 0.9 g and 60.65 g ± 0.95 g, respectively). Meanwhile, the survival rate of milkfish and mud crab was 95% ± 0.3% and 95 % ± 2.3%, respectively, followed by a lower FCR at T4 (1.54 ± 0.10). The water quality remained good for fish and mud crabs to survive. Milkfish and mud crab polyculture significantly affect the abundance of phytoplankton, demonstrating a good community structure.

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## REFERENCES

Agbayani, R. F. (2001). Production economics and marketing of mud crabs in the Philippines. *Asian Fisheries Science*, *14*(2), 201-210. https://doi.org/10.33997/j.afs.2001.14.2.010

Ali, M. A., Hossain, G. S., Biswas, M. M. R., Barman, S. K., & Huq, K. A. (2009). Polyculture and integrated culture pattern of freshwater prawn in fresh to hyposaline water. *International Journal of Sustainable Crop Production*, *4*(4), 23–27.

Araújo-Silva, S. L., Moraes, M. D. A. B., do Carmo, C. F., Osti, J. A. S., Vaz-dos-Santos, A. M., & Mercante, C. T. J. (2014). Effluent of a polyculture system (tilapias and shrimps): Assessment by mass balance of nitrogen and phosphorus. *Journal of Environmental Protection*, *5*(10), 797–802. https://doi.org/10.4236/jep.2014.510081

Asadujjaman, M., Biswas, S., Manirujjaman, M., Rahman, M., Hossain, M. A., & Islam, M.

A. (2015). Determination of protein, lipid and carbohydrate contents of conventional and non-conventional feed items used in carp polyculture pond. *Journal of Aquaculture Research and Development*, *6*(2), 1000301. https://doi.org/10.4172/2155-9546.1000301

Barman, U. K., Garg, S. K., & Bhatnagar, A. (2012). Effect of different salinity and ration levels on growth performance and nutritive physiology of milkfish, Chanos chanos (Forsskal) - Field and laboratory studies. *Fisheries and Aquaculture Journal*, *2012*, FAJ-53. https://doi.org/10.4172/2150-3508.1000053

Basmi, H. J. (2000). *Planktonologi: Plankton sebagai bioindikator kualitas perairan* [Planktonology: Plankton as a bioindicator of water quality]. Institut Pertanian Bogor.

Chopin, T. (2013). Aquaculture, integrated multi-trophic (IMTA). In P. Christou, R. Savin, B. A. Costa-Pierce, I. Misztal, & C. B. A. Whitelaw (Eds.), *Sustainable food production* (pp. 195-217). Springer. https://doi.org/10.1007/978-1-4614-5797-8_173

Davis, J. (2011). *Polyculture opportunities in the mid-hills of Nepal for resource poor farmers: Ecological aquaculture studies and reviews.* University of Rhode Island.

De-shang, L., & Shuang-lin, D. (2000). Summary of studies on closed-polyculture of penaeid shrimp with fishes and moluscans. *Chinese Journal of Oceanology and Limnology*, *18*, 61–66. https://doi.org/10.1007/BF02842543

Dolgov, A. V., & Prokopchuk I. P. (2018). Macrozooplankton of the Arctic - The Kara Sea in relation to environmental conditions: A comment on Dvoretsky and Dvoretsky (2017). *Estuarine, Coastal and Shelf Science*, *209,* 205-207. https://doi.org/10.1016/j.ecss.2018.05.010

Gaillard, J. (2010). *Development of the mud crab sector in three provinces of the Philippines – Constraints and prospects*. https://www.doc-developpement-durable.org/file/Elevages/crabes/development%20of%20the%20mud%20crab%20sector_Philippines.pdf

Ghosh, D., Sathianandan, T. V., & Vijayagopal, P. (2011). Feed formulation using linear programming for fry of catfish, milkfish, tilapia, Asian sea bass, and grouper in India. *Journal of Applied Aquaculture*, *23*(1), 85–101. https://doi.org/10.1080/10454438.2011.549781

Ihsan, Y. N. (2012). *Nutrient fluxes in multitrophic aquaculture systems* [Master's thesis, University of Kiel]. University of Kiel Repository. https://www.tierzucht.uni-kiel.de/de/forschung/dissertationen-1/diss_ihsan_12.pdf

Jamerlan, G. S., Coloso, R. M., & Golez, N. V. (2014). *Intensive culture of milkfish* Chanos chanos *in polyculture with white shrimp* Penaeus indicus *or mud crab* Scylla serrata *in brackishwater earthen ponds*. Aquaculture Department, Southeast Asian Fisheries Development Center.

Jaspe, C. J., Caipang, C. M. A., & Elle, B. J. G. (2011). Polyculture of white shrimp, *Litopenaeus vannamei* and milkfish, *Chanos chanos* as a strategy for efficient utilization of naturalfood production in ponds. *Animal Biology and Animal Husbandry*, *3*(2), 96-104.

Kwon, H. K., Kim, G., Lim, W. A., & Park, J. W. (2018). *In-situ* production of humic-like fluorescent dissolved organic matter during *Cochlodinium polykrikoides* blooms. *Estuarine, Coastal and Shelf Science*, *203*, 119-126. https://doi.org/10.1016/j.ecss.2018.02.013

Lall, S. P. (2000, 19-22 November). Nutrition and health of fish. In L. E. Cruz-Suárez, D. Ricque-Marie, M. Tapia-Salazar, M. A. Olvera-Novoa, & R. Civera-Cerecedo (Eds.), *Avances en Nutrición Acuícola V*. Memorias del V Simposium Internacional de Nutrición Acuícola, Mérida, Yucatán, Mexico (pp. 13-23).

Laxmappa, B., & Khrisna, S. M. (2015). Polyculture of the freshwater prawn *Macrobrachium*

*malcolmsonii* (H. M. Edwards) in Koilsagar reservoir of Mahabubnagar district (TS), India. *International Journal of Fisheries and Aquatic Studies*, *2*(4), 147–152.

Malleo, J. (2011). *Economics of mud crabs farming in Pangani: Is there significant income contribution to the coastal community?*. https://vdocuments.net/economics-of-mud-crabs-farming-in-economics-of-mud-crabs-farming-in-pangani.html?page=1

Martan E. (2008). Polyculture of fishes in aquaponics and recirculating aquaculture. *Aquaponics Journal*, *48*(1), 28–33.

Miroslav, C., Dejana, T., Dragana, L., & Vesna, D. (2011). Meat quality of fish farmed in polyculture in carp ponds in Republic of Serbia. *Technologija Mesa*, *52*(1), 67–68.

Monwar, M. M., Ruhul, A. K. M., Sarker, A., & Das, N. G. (2017). Polyculture of seabass with tilapia for the utilization of brown fields in the coastal areas of Cox's Bazar, Bangladesh. *International Journal of Fisheries and Aquaculture*, *5*(6), 104–109. https://doi.org/10.5897/IJFA2013.0347

Napiórkowska-Krzebietke, A. (2017). Phytoplankton as a basic nutritional source in diets of fish. *Journal of Elementology*, *22*(3), 831-841. https://doi.org/10.5601/jelem.2016.21.4.1375

Nunes, J. P., Ferreira, J. G., Gazeau, F., Lencart-Silva, J., Zhang, X. L., Zhu, M. Y., & Fang, J. G. (2003). A model for sustainable management of shellfish polyculture in coastal bays. *Aquaculture*, *219*(1–4), 257–277. https://doi.org/10.1016/S0044-8486(02)00398-8

Primavera, J. H. (2006). Overcoming the impacts of aquaculture on the coastal zone. *Ocean and Coastal Management*, *49*(9–10), 531–545. https://doi.org/10.1016/j.ocecoaman.2006.06.018

Samidjan, I., & Rachmawati, D. (2016). Effect of artificial feed on the growth and survival of white shrimp (*Litopenaeus vannamei*) and milkfish (*Chanos chanos*) in application of innovative polyculture technology. *Jurnal Teknologi*, *78*(4–2), 91–98. https://doi.org/10.11113/jt.v78.8187

Samidjan, I., & Rachmawati, D. (2018). Engineering technology of fish farming floating nets cages on polka dot grouper (*Cromileptes altivelis*) used artificial feed enriched phytase enzyme. In *IOP Conference Series: Earth and Environmental Science* (Vol. 116, No. 1, p. 012010). IOP Publishing. https://doi.org/10.1088/1755-1315/116/1/012010

Samidjan, I., Dody, S., & Rachmawati, D. (2020). Biodiversity of phytoplankton from polyculture milkfish and white shrimp vanname pond culture waters, Pekalongan region. In *IOP Conference Series: Earth and Environmental Science* (Vol. 530, No. 1, p. 012040). IOP Publishing. https://doi.org/10.1088/1755-1315/530/1/012040

Samidjan, I., Hutabarat, Y., Rachmawati, D., & Herawati, V. E. (2019). The effect of polyculture white shrimp vannamei and seaweed on different plant distance on growth, survival and phytoplankton abundance. *Aquacultura Indonesiana*, *20*(2), 57-71. https://doi.org/10.21534/ai.v20i2.140

Siskey, M., & Baldwin, R. (2011). *Integrated multi-trophic aquaculture - TECH 797*. https://nsgl.gso.uri.edu/nhu/nhut11004.pdf

Solomon, J. R., & Ezigbo, M. N. (2010). Polyculture of heteroclarias / tilapia under different feeding regimes. *New York Science Journal*, *3*(10), 42–57.

Spellerberg, I. F., & Fedor, P. J. (2003). A tribute to Claude Shannon (1916–2001) and a plea for more rigorous use of species richness, species diversity and the 'Shannon–Wiener' Index. *Global Ecology and Biogeography*, *12*(3), 177–179. https://doi.org/10.1046/j.1466-822X.2003.00015.x

Suhartono, S. & Istiyanto, S. (2014). Dynamic model development to improve grouper cultivation

production in floating net cages in Karimun Java islands, Jepara. *Journal of Applied Mathematical Sciences*, *8*(179), 8921–8928. https://doi.org/10.12988/ams.2014.48652

Sun, W., & Boyd, C. E. (2013). Phosphorus and nitrogen budgets for inland, saline water shrimp ponds in Alabama. *Fisheries and Aquaculture Journal*, *4*(1), 1000080. https://doi.org/10.4172/2150-3508.1000080

Tan, S. N., Teng, S. T., Lim, H. C., Kotaki, Y., Bates, S. S., Leaw, C. P., & Lim, P. T. (2016). Diatom *Nitzschia navis-varingica* (Bacillariophyceae) and its domoic acid production from the mangrove environments of Malaysia. *Harmful Algae*, *60*, 139-149. https://doi.org/10.1016/j.hal.2016.11.003

Ulfah, M., Fajri, S. N., Nasir, M., Hamsah, K., & Purnawan, S. (2019). Diversity, evenness and dominance index reef fish in Krueng Raya Water, Aceh Besar. In *IOP Conference Series: Earth and Environmental Science* (Vol. 348, No. 1, p. 012074). IOP Publishing. https://doi.org/10.1088/1755-1315/348/1/012074

Venugopal, G., Razvi, S. S. H., Babu, P. P. S., Reddy, P. R., Mohan, K. M., & Srinivasa, P. (2012). Performance evaluation of mud crab *Scylla serrata* (Forskal, 1775) in monoculture, monosex culture and polyculture. *Journal of the Marine Biological Association of India*, *54*(2), 5–8.

Xie, B., Jiang, W., & Yang, H. (2011). Growth performance and nutrient quality of Chinese shrimp *Penaeus chinensis* in organic polyculture with razor clam *Sinonovacula constricta* or hard clam *Meretrix meretrix*. *Bulgarian Journal of Agricultural Science*, *17*(6), 851–858.

Yang, Y., & Fitzsimmons, K. (2002). *Tilapia shrimp polyculture in Thailand*. Asian Institute of Technology.

Yuan, D., Yi, Y., Yakupitiyage, A., Fitzsimmons, K., & Diana, J. S. (2010). Effects of addition of red tilapia (*Oreochromis* spp.) at different densities and sizes onproduction, water quality and nutrient recovery of intensive culture of white shrimp (*Litopenaeus vannamei*) in cement tanks. *Aquaculture*, *298*(3-4), 226–238. https://doi.org/10.1016/j.aquaculture.2009.11.011

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# Soil Element Assessment in Organic Paddy Fields in the Thung Kula Ronghai Zone, Thailand

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## ABSTRACT

Organic rice production (ORP) has been promoted as a means of sustaining both farmers and the ecology of paddy fields, so this research aims to evaluate soil properties and soil elements in the ORP and general rice production (GRP) systems in the Thung Kula Ronghai (TKR) zone in Thailand. Soil samples were collected in Roi-et province from fields classified as ORP (5 fields) or GRP (4 fields), and interviews were also conducted with the field owner about rice yield and rice production. Data from the ORP and GRP groups were compared by *t*-test, and soil enhancement practices were measured by one-way analysis of variance (ANOVA) for variances. Results indicate there were 14 indicators of soil element control in the TKR. All indicators in the ORP and GRP systems were lower than the rate in soil that is suitable for rice production. The macroelement content in the TKR zone was total nitrogen > total potassium > phosphorus available at a ratio of 338: 3: 1, and the soil organic matter (SOM)/soil organic carbon (SOC) ratio is about 3.45. The soil improvement techniques used in the ORP systems—manure only and manure combined with green manure—have a higher pH value ($p < 0.05$) than the fertilizer only input but a lower TK value ($p < 0.05$) than the fertilizer only input. As a result, the ORP yield was higher than that of the GRP systems ($p < 0.05$), greatly affecting farmers' practices.

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## INTRODUCTION

The organic rice production (ORP) system in Thailand promotes farmers' health, increases the sustainability of ecological systems, and produces a higher value crop

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(Ueasangkomsate et al., 2018). In addition to being ecologically friendly, ORP can increase biodiversity in the fields (Avasthe et al., 2018; Rahmann, 2011; Reeve et al., 2016) because organic fertilizer and organic pesticides control rice production process (Lin & Fukushima, 2016). It is the reason the government is trying to include this system in its development program that aims to increase the ORP area in the country (Herique & Faysse, 2020). Thailand's rice-producing area is about 9.5 million ha, and 61% of paddy fields are in the country's northeast region, making this a significant area for rice production (Office of Agricultural Economics [OAE], 2019). ORP represents more than 80% of all rice grown in the northeast region of Thailand (Thuansri & Morathop, 2016).

The northeast region of Thailand is a major area of high-quality ORP. The Thung Kula Ronghai (TKR) zone is located on the Khorat plateau. It has an area of about 320,000 ha covering 13 districts in 5 provinces: (1) Phayakaphum Phisai district in Maha Sarakham province, (2) Champhonburi and Thatum districts in Surin province, (3) Phatumrat, Kasetwisai, Suwannaphumdi, Phonsai, and Nonghee districts in Roi-et province, (4) Sira-art, Rasisarai, and Yangchumnoi districts in Srisaket province, and (5) Khorwang and Mahachanachai districts in Yasothon province. Approximately 70%, or 224,000 ha, of the TKR zone is used for rice production, representing 3.96% of the country's northeast region. In the past, the TKR zone experienced problems with soil fertility because the soil in the region is sandy loam and silty clay; therefore, it does not retain moisture, rendering the soil less fertile (Loeffler et al., 1993; Sompob, 1986). However, the situation did not affect the quality of rice grown in the area (Saetung & Trelo-ges, 2017), and rice produced in TKR is well known domestically and internationally.

Considering how ORP affects the nutrient balance in the soil has led to the research question of whether there is a difference in soil element in the ORP and general rice production (GRP) systems in the TKR zone. The purpose of this study was to evaluate soil properties and soil elements in ORP and GRP systems to support continuing farmer discussions about selecting rice production systems in TKR. When evaluating soil elements in paddy fields, indicators should be considered. Soil organic matter (SOM) is one indicator of soil fertility, as are soil organic carbon (SOC), soil pH, carbon/nitrogen (C/N) ratio, nitrogen (N), phosphorus (P), available potassium (K), and electrical conductivity (EC) (Khaki et al., 2017; Supriyadi et al., 2017). This information can develop soil improvement techniques to increase ORP in the TKR zone.

## MATERIALS AND METHODS

### Soil Collection

The TKR study site in Roi-et province comprised nine plots distributed across two districts—five organic paddy fields in Phatumrat district and four general paddy fields in Kasetwisai district. The soil

samples were collected from eight points in a Z shape (shown as red stars in Figure 1) for mixing and were placed in plastic bags for element analysis. Two levels of topsoil (0–5 cm and 5–20 cm) were used (shown as back dots in Figure 1) to measure bulk density and biomass. The soil was collected by soil core, stored in plastic bags, and kept in an icebox.

## Field Study Experiment

TKR1 to TKR5 are organic fields fertilized with manure prior to plowing. Organic fertilizer was applied 2–5 weeks after rice planting and 12–16 weeks after planting. Farmers also used a bio-extract hormone supplement during the rice production process (spraying 7–12 weeks after planting). Different methods were used in the ORP system: TKR3, TKR4, and TKR5 were treated with green manure either after harvest or before planting, but TKR1 and TKR2 did not have the green manure input. Before plowing in the GRP system, farmers used cow and chicken manure in the fields. After planting, the farmer applied fertilizer twice: first, at the early rice-growing stage (about 4–6 weeks after broadcasting) using a formula of 16-16-8 (% of nitrogen [N], phosphorus [P], and potassium [K]) at a rate of 50 kg/ha; and second, at the early grain production stage (about 12–15 weeks after broadcasting) using a formula of 15-15-0 at a rate of 62.5 kg/ha. In addition, in TKR_1 and TKR_4, straw was burned after the rice was harvested.

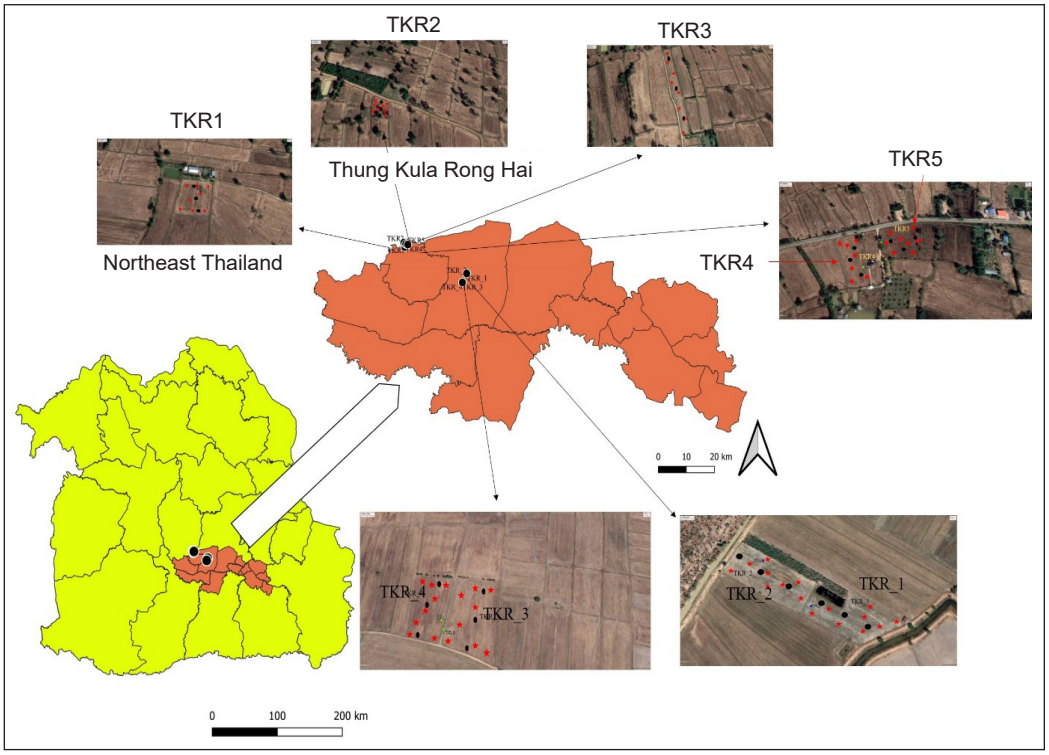


*Figure 1*. Study site and field plots where soil samples were collected

**Physical Survey of Soil in the Field**

Soil moisture levels were measured during the dry season in March 2021 using a Delta-T Devices series HH2 moisture meter (United Kingdom). This multi-sensor instrument auto-detects the amount of moisture in the soil (%), soil temperature, and soil EC as well as can measure the soil surface to a depth of 5 cm. It also determines soil color, which was used to confirm soil type using the Munsell Soil Color Book.

The pH value and sodium chloride (NaCl) content of the soil were tested using a solution technique. The soil sample was dissolved in water at a 1:2 ratio of 5 g of soil diluted in 10 mL of deionized water, and shaken for 30 min. After waiting an additional 30 min to allow for precipitation, the liquid was separated from the sample for pH and NaCl content checking using a Hach HQ40d portable multimeter (USA). EC was checked via a solution technique using electrochemistry instruments from the EUTECH CON700 series (USA).

**Soil Extraction and Element Analysis**

The collected soil samples were placed in plastic bags and kept in an icebox while transported from the field to the laboratory. The soil was dried in a 105°C oven for 72 hours, then ground using a mortar and pestle. Net No. 4 (10 mm) of sifted soil were selected and maintained in the refrigerator at a temperature of 4°C.

The soil extraction used in AAS analysis was a 2 g soil sample with concentrated nitric acid ($HNO_3$) and concentrated perchloric acid ($HClO_4$) (1:1) for 10 mL (United States Environmental Protection Agency [US EPA], 1996). It was then digested at about 500°C in the SpeedDigester K-425 BUCHI until dried (Switzerland). Each residue was rinsed with 1% $HNO_3$ then sieved through Whatman No.1 paper. The supernatant was then transferred to a 50 mL volumetric flask, and 1% $HNO_3$ was added for continued atomic absorption spectrophotometers (AAS) analysis (Thummahitsakul et al., 2018).

The analysis of nitrogen and carbon formed total nitrogen (TN) and total carbon (TC) in the samples analyzed by the LECO series CHN-628 CHN Analyzer (USA). Potassium (K) analysis was performed using AAS, an Agilent series 240AA instrument (USA). Mineral content analysis and the level of phosphorus (P) available in the soil content were analyzed using the Bray II method (Bray & Kurtz, 1945) and measured by spectrophotometers at a wavelength of 882 (nm).

Jenkinson and Powlson's (1976) technique was applied to prepare the soil for biomass analysis. A 20 g soil sample was incubated for about 72 hours in polyethylene bags, after which it was dried in a 105°C oven for 24 hours and placed into glass beakers (10 g) for fumigation with chloroform ($CHCl_3$) in desiccators for 72 hours. A CHN-628 CHN analyzer (USA) was used to analyze the percentage of carbon content in the soil.

### Statistical Analysis

The data were analyzed by t-test in $p < 0.05$ using data components of the ORP and GRP systems in the TKR zone, such as rice yield production and quantity of element in the soil. However, the soil improvement practices were determined using one-way ANOVA for variances. In addition, differences in data were compared using post-hoc Tukey's honestly significant difference (HSD) in $p < 0.05$. Finally, all analyses used the SPSS V.22 and Sigmaplot 12.0 (free trial).

## RESULTS

### Soil Properties

The paddy fields of TKR are made up of sandy soil, as confirmed by the Munsell Soil Color Book. The soil contains the mineral goethite, its texture is very fine, and its color is different from the plots where the soil samples were collected so that the same sets of soils characteristic Ki series in the USDA classification are fine loamy and isohyperthemic typic natraqualfs types (Land Development Department, 2021). The soil pH of ORP systems averaged 5.6 ± 0.32, which is significant ($p < 0.01$), while the GRP systems had an average pH of 4.74 ± 0.26. However, the percentage of NaCl in the soil in ORP systems averaged 0.22% ± 0.12%; the percentage in GRP systems averaged 0.27% ± 0.31%. The EC in ORP systems averaged 252.74 ± 122.12, and the EC in GRP systems averaged 359.40 ± 297.28. The bulk density of the soil surface (0–5cm; BD5) in ORP systems averaged 0.39 ± 0.18 g/cm$^3$; GRP systems had an average bulk density of 0.27 ± 0.15 g/cm$^3$. At a depth of 6–20 cm (BD20), the soil bulk density averaged 0.99 ± 0.43 g/cm$^3$ in ORP systems and 0.80 ± 0.26 g/cm$^3$ in GRP systems.

### Soil Moisture

In the field survey, dry conditions prevented the soil moisture volume from being collected; daytime temperatures reached a high of 35.56°C ± 2.53°C. However, collected soil samples dried in a 105°C oven for three days were found to have topsoil (0-5cm) moisture content of 1.46% ± 0.72%. At 6–20 cm soil depth, the soil had a moisture level of 3.67% ± 1.4%. In ORP systems, the average topsoil temperature was 35.72°C ± 3.74°C, and the soil moisture level averaged 1.81% ± 0.71% for topsoil and 4.37% ± 1.4% for soil at a depth of 6–20 cm. In GRP systems, the topsoil had an average temperature of 35.37°C ± 0.75°C and an average soil moisture level of 1.03% ± 0.69% for topsoil and 2.80% ± 0.81% for soil at a depth of 6–20 cm. Differences between the three indicators—temperature, soil moisture percentage of topsoil, and a soil moisture percentage of soil 6–20 cm deep—in the ORP and GRP groups were not significant, present in Table 1. The correlation between temperature and soil moisture at a depth of 6–20 cm ($r = 795$; $p < 0.05$) is shown in Figure 2. However, the soil moistures will decrease to temperature increasing (Tang & Chen, 2017) related to temperature are indicated with performing of agriculture yield production (Rahman

et al., 2020), because the parameter has impacted to microorganism activity in the soil, so the soil moisture is better to microorganism activity has about 30-40% of soil moisture and temperature to better with microbial activity about 20-40°C (Cruz-Paredes et al., 2021).

Table 1
*Soil moisture and temperature data from the field survey in TKR*

| Field | Pattern of rice production | % moisture of topsoil (0-5 cm) | % moisture of soil (6-20 cm) | Temperature (°C) |
|---|---|---|---|---|
| TKR1 | ORP | 2.32 | 2.40 | 30.1 |
| TKR2 | ORP | 1.10 | 2.61 | 33.6 |
| TKR3 | ORP | 2.18 | 5.21 | 38.3 |
| TKR4 | ORP | 0.981 | 5.23 | 38.3 |
| TKR5 | ORP | 2.46 | 6.40 | 38.3 |
| | Average | 1.81 | 4.37 | 35.72 |
| | SD | 0.710 | 1.77 | 3.74 |
| TKR_1 | GRP | 1.74 | 2.38 | 34.5 |
| TKR_2 | GRP | 0.211 | 1.99 | 35 |
| TKR_3 | GRP | 0.740 | 2.96 | 36 |
| TKR_4 | GRP | 1.45 | 3.86 | 36 |
| | Average | 1.03 | 2.80 | 35.37 |
| | S.D. | 0.693 | 0.81 | 0.75 |

*Note*. TKR = Thung Kula Ronghai; ORP = Organic rice production; GRP = General rice production; S.D. = Standard deviation

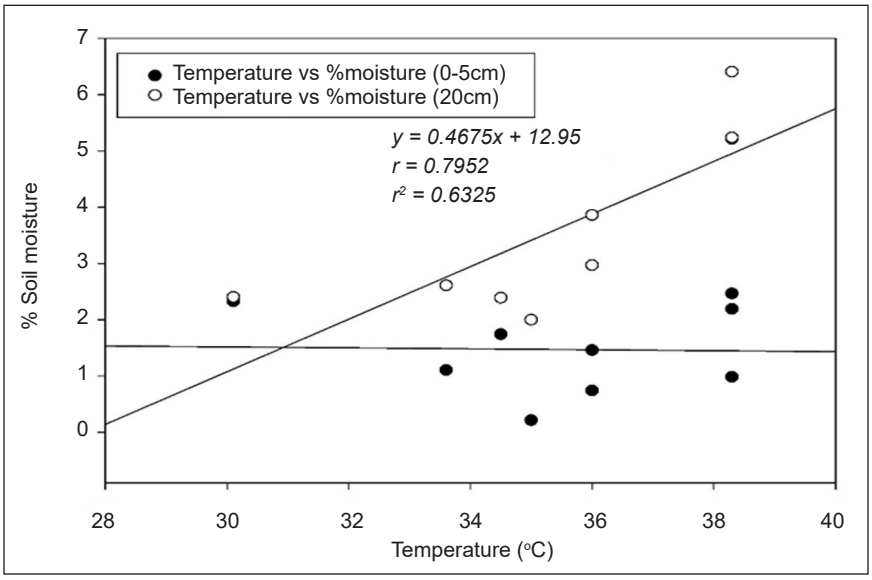


*Figure 2*. Correlation between soil surface temperature and soil moisture percentage at a depth of 6–20 cm in TKR during the dry season

## Rice Yield Production in the Field Survey

Interviews with the paddy field owner found that between 2017 and 2020, the plot TKR5 had a higher yield production (3093.7 ± 759.4 kg/ha). In 2018, TKR1, TK2, and TK3 cannot be harvested because the dough affects the farmer's yield loss product. However, TKR5 was used for glutinous rice cultivation, while TKR 1, 2, 3, and 4 produced the Hom Mali 105 (jasmine rice) variety and the yield production of the present in Table 2. However, when considering with quantity, rice yield of ORP was found to average 507 (±127) kg/ha and the production of GRP average 238 (±51) kg/ha (Table 3), so that rice production yields for organic and general rice production were significant ($p < 0.01$), indicating that organic paddy fields produce higher yields than general paddy fields (Figure 3).

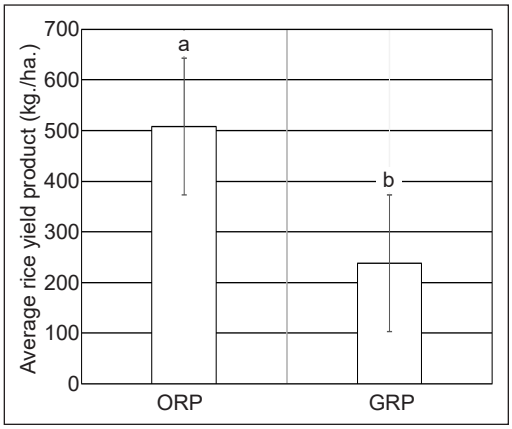


*Figure 3*. Average the rice yield production at the study site comparison of rice yield production among organic and general rice production systems in TKR ($p < 0.01$)

Table 2
*Rice production quantities from 2017 to 2020 (kg/ha)*

| Year | TKR1 | TKR2 | TKR3 | TKR4 | TKR5 | TKR_1 | TKR_2 | TKR_3 | TKR_4 | $\bar{X}$ of ORP | $\bar{X}$ of GRP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2020 | 2437 | 3409 | 4687 | 3333 | 3750 | 1562 | 1687 | 1015 | 1458 | 3523 | 1430 |
| 2019 | 3062 | 3409 | 4687 | 2500 | 3750 | 1770 | 1964 | 1273 | 1718 | 3481 | 1681 |
| 2018 | 0 | 0 | 0 | 2187 | 2500 | 1437 | 1517 | 1328 | 1302 | 937 | 1396 |
| 2017 | 2875 | 2272 | 2500 | 1968 | 2375 | 2187 | 1071 | 1406 | 1031 | 2398 | 1424 |
| $\bar{X}$ | 2093 | 2272 | 2968 | 2497 | 3093 | 1739 | 1560 | 1255 | 1377 | 2585 | 1483 |
| S.D. | 1420 | 1607 | 2231 | 598 | 759 | 328 | 374 | 169 | 287 | 607 | 1215 |

*Note*. Fields TKR1, TKR2, and TKR3 to 2018 cannot be harvested due to drought; ORP = Organic rice production are field TKR1, TKR2, TKR3, TKR4, and TKR5.; GRP = General rice production are TKR_1, TKR_2, TKR_3, and TKR_4

Table 3
*Compares rice quantity between organic and general rice production system in TKR*

| | *t* | df | Sig. (2-tailed) | Mean difference |
|---|---|---|---|---|
| Organic rice production systems | 15.395 | 14 | 0.00 | 507.616 |
| General rice production system | 20.859 | 19 | 0.00 | 238.289 |

*Note*. The mean difference is significant at the *p*-value < 0.05 level

## Element and Mineral Quantities in Organic and General Paddy Fields

The quantity of essential elements in the soil content in TKR is as follows: TN, approximately 210 mg/kg; TK, approximately 2.06 mg/kg; and available P, approximately 0.62 mg/kg. Therefore, the ratio of TN > TK > available P is 338:3:1. The ORP TN value averaged 209 ± 2.57 mg/kg, while the GRP value averaged 210 ± 2.40 mg/kg to compare the organic and general groups. Available P in ORP systems averaged 0.825 ± 0.391 mg/kg; available P in GRP systems averaged 1.76 ± 1.18 mg/kg. The TK level in GRP soil content averaged 2.65 ± 0.15 mg/kg, which is significant ($p < 0.01$), the TK level in ORP soil content averaged 1.47 ± 0.18 mg/kg, present in Table 4. The amounts of macroelements in ORP and GRP are illustrated in Figure 4. The soil element in assessing TKR is mineral content (TN, P available, and TK), soil pH, EC, percentage of sodium chloride, bulk density of topsoil, and soil deep 6-20 cm, soil organic matter, and soil organic carbon of topsoil and soil deep 6–20 cm is provided in Table 5.

Table 4
*Soil element content and comparison of soil macro-elements between the organic production system and the general rice production system in TKR*

| Item | Unit | ORP | GRP | *t* | Sig.(2-tailed) |
|---|---|---|---|---|---|
| N | mg./kg | 209 (±2.57) | 210 (±2.40) | 0.011 | 0.991 |
| P | mg./kg | 0.825 (±0.391) | 1.76 (±1.18) | -1.189 | 0.319 |
| K | mg./kg | 1.47 (±0.186) | 2.65 (±0.152) | -13.09 | 0.00 |

*Note*. The mean difference is significant at the *p*-value < 0.05 level.; P was determined to use the Bray II method. The phosphorus considered P available from potassium dihydrogen phosphate ($KH_2PO_4$), N was total nitrogen, and K was total potassium.; ORP = Organic rice production system; GRP = General rice production system

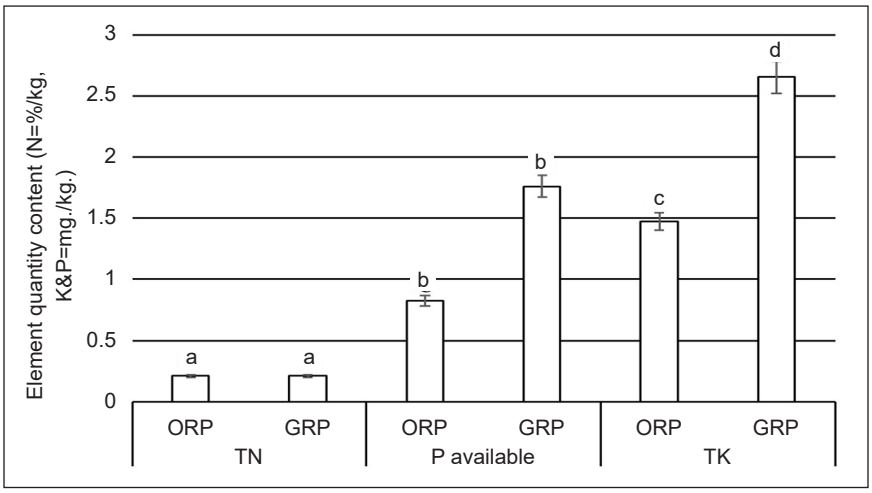


*Figure 4*. Quantity of macroelements comparison of ORP and GRP in TKR found to TK between ORP and GRP significant ($p < 0.01$)

Table 5
*Volume of parameter of studies site of TKR*

| Item | Unit | TKR1 | TKR2 | TKR3 | TKR4 | TKR5 | TKR_1 | TKR_2 | TKR_3 | TKR_4 | Average | SD | SE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TC | g/kg | 3.23 | 2.21 | 2.21 | 2.21 | 6.75 | 3.61 | 3.66 | 6.26 | 1.68 | 3.53 | 1.82 | .607 |
| TN | g/kg | 0.20 | 0.21 | 0.21 | 0.21 | 0.20 | 0.213 | 0.21 | 0.20 | 0.20 | .21 | .002 | .001 |
| P available | mg./kg | 0.85 | 1.33 | 1.05 | 0.44 | 0.43 | 0.743 | 0.73 | 2.83 | 2.73 | 1.24 | .919 | .306 |
| TK | mg./kg | 1.59 | 1.40 | 1.18 | 1.65 | 1.53 | 2.8815 | 2.56 | 2.56 | 2.61 | 1.99 | .643 | .214 |
| pH* | | 5.22 | 5.6 | 5.97 | 5.82 | 5.02 | 4.36 | 4.82 | 4.88 | 4.93 | 5.18 | .523 | .174 |
| EC | (µS-1) | 357 | 325 | 84 | 244 | 159 | 759 | 170 | 407 | 100 | 289 | 209 | 69.9 |
| NaCl | % | 0.2 | 0.16 | 0.4 | 0.12 | 0.09 | 0.73 | 0.09 | 0.21 | 0.05 | .22 | .214 | .071 |
| BD (5) | g/dm3 | 0.59 | 0.26 | 0.49 | 0.20 | 0.68 | 0.386 | 0.04 | 0.27 | 0.37 | .36 | .199 | .066 |
| BD (20) | g/dm4 | 0.55 | 0.75 | 1.54 | 1.13 | 2.12 | 0.591 | 0.56 | 0.96 | 1.08 | 1.03 | .524 | .174 |
| C/N ratio | | 15.6 | 10.5 | 10.4 | 10.4 | 32.6 | 16.9 | 17.3 | 30.1 | 8.04 | 16.9 | 8.85 | 2.95 |
| SOM (5) | mg./kg | 1242 | 852 | 850 | 852 | 2598 | 1389 | 1408 | 2408 | 646 | 1360 | 701 | 233 |
| SOC (5) | mg./kg | 360. | 247 | 246 | 247 | 753 | 402 | 408 | 698 | 187 | 394 | 203 | 67.7 |
| SOM (20) | mg./kg | 1002 | 642 | 650 | 592 | 2308 | 1149 | 1168 | 2168 | 406 | 1120 | 685 | 228 |
| SOC (20) | mg./kg | 290 | 186 | 188 | 171 | 669 | 333 | 338 | 628 | 117 | 325 | 198. | 66.3 |

*Note.* * Testing in water soluble ; TC = Total carbon; TN = Total nitrogen; P available = Phosphorus available; TK = Total potassium; EC = Electrical conductivity; NaCl = Sodium chloride; BD (5) = Bulk density of top soil; BD (20) = Bulk density deep 20 cm.; C/N ratio = Ratio between carbon and nitrogen; SOM (5) = Soil organic matter of top soil; SOC (5) = Soil organic carbon of top soil; SOM (20) = Soil organic matter of soil deep 20 cm; SOC (20) = Soil organic carbon of soil deep 20 cm

### Essential Soil Element Assessment in ORP Systems in TKR

The value of TN and available P in the paddy fields of TKR in ORP and GRP systems was not significant: TN was approximately 0.20 and 0.21 g/kg, respectively, and available P was approximately 0.825 and 1.762 mg/kg, respectively. The TK value in ORP was lower than ($p < 0.05$) that of the GRP system, at approximately1.473, and 2.655 mg/kg, respectively, so the value of essential elements in ORP and GRP systems in the TKR zone is less than the quantities detailed in Arunrat et al. (2020)'s report. This report found the following essential mineral content in paddy fields in the tropical monsoon region: TN, approximately 0.41 g/kg; available P, approximately 2.77 mg/kg; and TK, approximately 56.71 mg/kg. However, the quantity of essential elements in the soil content in ORP tends to be lower than in GRP. It is similar to Islam et al. (2017)'s and Kakar et al. (2020)'s findings that areas that use only organic manure (animal manure, sawdust, and vermicompost) have lower quantities of essential elements in the soil than areas that use chemical fertilizer or chemical fertilizer combined with organic fertilizer. Major natural sources of TK, such as humus or rice straw, can increase potassium levels, but their use should be limited to no more than 120 days (Li et al., 2014) because microorganisms will digest the raw material until it is changed to humus and potassium oxide ($K_2O$) or K in the soil. This technique for increasing K in the soil cannot be used in TKR because the dry climate and high temperature affect the ability of microorganisms and earthworms to digest humus (Möller, 2015; Pathma & Sakthivel, 2012), so the value of soil indicators present in Table 6.

### Volume of Biomass Content in Organic and General Paddy Fields

The SOM content of the soil surface (SOM5) in ORP systems had an average value of 1279.3 mg/kg, and the GRP value averaged 1463.04 mg/kg. The value of SOM in SOM20 in ORP averaged 1039.30 mg/kg, and the value in GRP was approximately 1223.04 mg/kg; there were no significant differences ($p > 0.05$). The value of the SOC content of the soil surface (SOC5) in ORP averaged 340.99 mg/kg, and the value in GRP averaged 424.28 mg/kg. The value of SOC in SOC20 in ORP averaged 301.39 mg/kg, and the GRP value was approximately 354.68 mg/kg; there were no significant differences. The C/N ratio in ORP had an average value of 15.93, and the GRP value averaged 18.10. The SOM, SOC, and C/N ratio values are presented in Table 7.

### Types of Soil-improving Activity

There were three methods of soil improvement used in the study: (1) manure only input, such as cow dung and chicken excrement; (2) manure combined with green manure input; and (3) fertilizer input. Of the 14 indicators—TC, TN, available P, TK, pH, EC, % NaCl, BD5, BD20, C/N ratio, SOM content of the soil surface at a depth of 0–5 cm (SOM5), SOC content of the soil surface at a depth of 0–5 cm (SOC5), SOM content of the soil surface at a depth of

Table 6
*The value of soil indicators in ORP and GRP systems in TKR compared with soil conditions suitable for rice production*

| Indicators | Soil in paddy fields in TKR | | Rate in soil suitable for rice production (Reference rate) | Reference of indicator |
|---|---|---|---|---|
| | ORP | GRP | | |
| TN (g/kg) | 0.209 | 0.210 | 0.93–0.52** | Araragi et al. (1978) |
| P available (mg/kg) | 0.825 | 1.762 | >15 | Saenya et al. (2015) |
| TK (mg/kg) | 1.473 | 2.655 | >20 | Saenya et al. (2015) |
| pH | 5.526 | 4.747 | >4.3 | Saenya et al. (2015) |
| EC ($\mu S^{-1}$) | 234.038 | 359.402 | <200 | Saenya et al. (2015) |
| % NaCl | 0.194 | 0.27 | | |
| Temperature (°C) | 36.92 | 35.375 | 25–38 | Saenya et al. (2015) |
| BD5 ($g/cm^3$) | 0.450 | 0.268 | 1.1–1.2/1.6 | Saenya et al. (2015); Zhou et al. (2014) |
| BD20 ($g/cm^3$) | 1.223 | 0.801 | 1.1–1.4/1.6 | Saenya et al. (2015); Zhou et al. (2014) |
| C/N ratio | 15.937 | 18.101 | 11.18** | Araragi et al. (1978) |
| SOM5 (mg/kg) | 1279.307 | 1463.048 | 14000–16000* | Saenya et al. (2015) |
| SOC5 (mg/kg) | 370.999 | 424.283 | 2000–3000/>150 | Ross (1993); Saenya et al. (2015) |
| SOM20 (mg/kg) | 1039.307 | 1223.048 | 14000–16000* | Saenya et al. (2015) |
| SOC20 (mg/kg) | 301.399 | 354.683 | 2000–3000* | Ross (1993) |

*Note*. *Used similar rate to topsoil (0–5 cm) because Ross (1993) and Saenya et al. (2015) reported the SOC value of the soil surface at a depth of 0–20 cm; ** Using low humic gley soil; TN = Total nitrogen; P available = Phosphorus available; TK = Total potassium; EC = Electrical conductivity; %NaCl = Percentage of sodium chloride in soil; BD5 = Bulk density of top soil; BD20 = Bulk density deep 20 cm.; C/N ratio = Ratio between carbon and nitrogen; SOM5 = Soil organic matter of top soil; SOC5 = Soil organic carbon of top soil; SOM20 = Soil organic matter of soil deep 20 cm; SOC20 = Soil organic carbon of soil deep 20 cm

Table 7
*The volume of SOM, SOC, and C/N ratio in ORP and GRP in TKR*

| Value | C/N ratio | | SOM5 (mg/kg) | | SOC5 (mg/kg) | | SOM20 (mg/kg) | | SOC20 (mg/kg) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ORP | GRP | ORP | GRP | ORP | GRP | ORP | GRP | ORP | GRP |
| $\bar{X}$ | 15.93 | 18.10 | 1279.30 | 1463.04 | 370.99 | 424.28 | 1039.30 | 1223.04 | 301.39 | 354.68 |
| S.D. | 9.61 | 9.07 | 756.69 | 723.09 | 219.44 | 209.69 | 728.17 | 723.09 | 211.17 | 209.69 |

*Note*. The value of C/N ratio, SOM5, SOC5, SOM20, and SOC20 indicators compares between ORP and GRP by *t*-test found to not significant ($p > 0.05$); C/N ratio = Ratio between carbon and nitrogen; SOM5 = Soil organic matter of top soil; SOC5 = Soil organic carbon of top soil; SOM20 = Soil organic matter of soil deep 20 cm; SOC20 = Soil organic carbon of soil deep 20 cm; ORP = Organic rice production; GRP = General rice production

6–20 cm (SOM20), and SOC content of the soil surface at a depth of 6–20 cm (SOC20) testing variances by one-way ANOVA in 3 were significant ($p < 0.05$): TK, pH, and BD20., this is shown in Table 8, and the correlation of all indicators found with the pH and TK values ($r$ = -0.855; $p < 0.05$), and the soil temperature and BD20 ($r$ = 0.755; $p < 0.05$), and EC and percentage of NaCl in soil ($r$ = 0.741; $p < 0.05$), also the soil organic matter group are C/N ratio, SOC, and SOM of the soil surface and deep soil 6-20 cm, shown in Table 9.

Table 8
*The value of indicator significance in soil improvement methods in TKR*

| Indicators | Manure | Manure + Green Manure | Fertilizer |
|---|---|---|---|
| TK (mg/kg) | 1.498[a] | 1.457[a] | 2.65[b] |
| pH | 5.41[a] | 5.6[a] | 4.47[b] |
| BD20 | 0.65[a] | 1.6[b] | 0.8[a] |

*Note*. [a,b] = The mean difference is significant at the $p$-value < 0.05 level.

### Effects of Burning Fields after Harvest

When rice fields burned after the harvest were tested using the *t*-test method, of the 14 indicators, the EC value was significant ($p < 0.05$) compared to the unburned fields. The EC value for burned fields averaged 583 ± 248 µS, while the value for unburned fields averaged 205 ± 106 µS. When considering the correlation of EC to other indicators in ORP and GRP, the EC value related to % NaCl ($r$ = 0.741) in ORP was significant ($p < 0.05$), as presented in Figure 5. However, opposite results were observed for the EC value in GRP. There was no significant correlation between EC and other components in the soil in the TKR fields.

## DISCUSSION

### ORP Activities to Reduce Soil Salinity and pH

The EC value is an indicator of soil health (United States Department of Agriculture

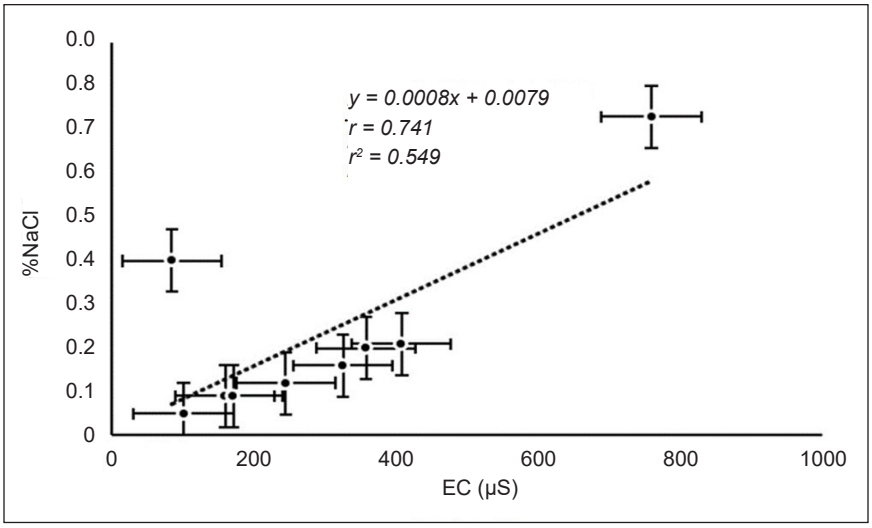


*Figure 5*. Correlation between EC and % NaCl in paddy field soil in ORP systems in TKR

Table 9
*The correlation of soil parameters in TKR*

| | TC | TN | P | TK | pH | EC | NaCl | Temp. |
|---|---|---|---|---|---|---|---|---|
| TC | 1 | -.535 | .010 | .156 | -.450 | .176 | -.041 | .165 |
| TN | -.535 | 1 | -.299 | .175 | .099 | .325 | .546 | -.244 |
| P | .010 | -.299 | 1 | .422 | -.198 | -.072 | -.193 | -.244 |
| TK | .156 | .175 | .422 | 1 | -.855** | .456 | .235 | -.471 |
| pH | -.450 | .099 | -.198 | -.855** | 1 | -.550 | -.281 | .480 |
| EC | .176 | .325 | -.072 | .456 | -.550 | 1 | .741* | -.526 |
| NaCl | -.041 | .546 | -.193 | .235 | -.281 | .741* | 1 | -.208 |
| Temp | .165 | -.244 | -.244 | -.471 | .480 | -.526 | -.208 | 1 |
| BD5 | .280 | -.558 | -.168 | -.437 | .052 | -.042 | .151 | .433 |
| BD20 | .376 | -.350 | -.124 | -.469 | .316 | -.527 | -.229 | .775* |
| C/N ratio | 1.000** | -.550 | .014 | .146 | -.442 | .164 | -.054 | .173 |
| SOM5 | 1.000** | -.535 | .010 | .156 | -.450 | .176 | -.041 | .165 |
| SOC5 | 1.000** | -.535 | .010 | .156 | -.450 | .176 | -.041 | .165 |
| SOM20 | 1.000** | -.532 | .021 | .154 | -.446 | .180 | -.030 | .154 |
| SOC20 | 1.000** | -.532 | .021 | .154 | -.446 | .180 | -.030 | .154 |

| | BD5 | BD20 | C/N ratio | SOM5 | SOC5 | SOM20 | SOC20 |
|---|---|---|---|---|---|---|---|
| TC | .280 | .376 | 1.000** | 1.000** | 1.000** | 1.000** | 1.000** |
| TN | -.558 | -.350 | -.550 | -.535 | -.535 | -.532 | -.532 |
| P avai. | -.168 | -.124 | .014 | .010 | .010 | .021 | .021 |
| TK | -.437 | -.469 | .146 | .156 | .156 | .154 | .154 |
| pH | .052 | .316 | -.442 | -.450 | -.450 | -.446 | -.446 |
| EC | -.042 | -.527 | .164 | .176 | .176 | .180 | .180 |
| NaCl | .151 | -.229 | -.054 | -.041 | -.041 | -.030 | -.030 |
| Temp | .433 | .775* | .173 | .165 | .165 | .154 | .154 |
| BD5 | 1 | .559 | .290 | .280 | .280 | .277 | .277 |
| BD20 | .559 | 1 | .384 | .376 | .376 | .369 | .369 |
| C/N ratio | .290 | .384 | 1 | 1.000** | 1.000** | .999** | .999** |
| SOM5 | .280 | .376 | 1.000** | 1 | 1.000** | 1.000** | 1.000** |
| SOC5 | .280 | .376 | 1.000** | 1.000** | 1 | 1.000** | 1.000** |
| SOM20 | .277 | .369 | .999** | 1.000** | 1.000** | 1 | 1.000** |
| SOC20 | .277 | .369 | .999** | 1.000** | 1.000** | 1.000** | 1 |

*Note*. * = Correlation is significant at the 0.05 level (2-tailed); ** = Correlation is significant at the 0.01 level (2-tailed); TC = Total carbon; TN = Total nitrogen, P avai.= Phosphorus available; TK = Total potassium; EC = Electrical conductivity; NaCl = Sodium chloride; BD5 = Bulk density of top soil; BD20 = Bulk density deep 20 cm.; C/N ratio = Ratio between carbon and nitrogen; SOM5 = Soil organic matter of top soil; SOC5 = Soil organic carbon of top soil; SOM20 = Soil organic matter of soil deep 20 cm; SOC20 = Soil organic carbon of soil deep 20 cm

[USDA], 2011) because it measures the salinity of the soil and is related to the ion exchange and soil pH. The study found that burning straw in the paddy fields after harvest influenced the EC value; the EC rate was higher in burned fields than in unburned fields ($p < 0.05$). The EC ratio of % NaCl in the soil ($r = 741$; $p < 0.05$) is presented in Table 7. Saline soil is a problem in the TKR zone (Secretariat of the Senate, 2001). Farmers in ORP systems use tilling or plowing straw as a soil improvement method. It is a technique for soil conservation (Freitas, 2000) that can lead to decreased soil erosion and increased organic carbon in the soil (Chen et al., 2019).

**Assessment of the SOM/SOC Ratio in ORP Systems in TKR**

The value of SOM can be attributed to major amendments in the soil (Swift, 1996) because SOM is related to microbial activity in the soil (Cynthia et al., 2016; Powlson et al., 2001). If the SOM in the soil is less than 1% (> 10 g/kg), fertilizer input should be used for soil amendment (Haque et al., 2021). The SOM5 value of the soil surface in the ORP and GRP systems in TKR averaged 0.13% and 0.15%, respectively, which is very low. GRP systems used manure combined with chemical fertilizer, while ORP systems used manure combined with green manure; however, soil element was not significantly improved in the ORP systems. In addition, the SOM value in ORP systems was below the ideal rate needed for rice production, about 14000–16000 mg/kg (Saenya et al., 2015). Therefore, the SOM value in GRP systems is suitable for rice production in the TKR zone.

SOM values are calculated using SOC content, which affects the indicators; the SOM/SOC ratio will consider the pedogenesis and degree of decomposition of organic and mineral soil substrate (Bianchi et al., 2008; Klingenfuß et al., 2014). In the paddy fields of TKR, the SOM/SOC ratio of topsoil is about 3.45/1. The convention factor of topsoil's SOM/SOC ratio should be more than 1.72, so its median value is 1.9 (Pribyl, 2010). The SOM/SOC ratio in TKR indicates that organic carbon levels in the soil are lower than what is suitable for growing rice (see Table 5), so farmers should adopt methods that increase organic carbon in the soil, such as including pasture in the fields or applying green manure to paddy fields.

**Evaluation Including Paddy Field Element Between ORP and GRP System in TKR**

The indicator of TN, P available, and TK in TKR found one element of significance is the TKR content in the soil of GRP high than ORP system ($p < 0.05$). Thus, in the ORP system will, the soil, improve by animal manure and green manure for adding TN and phosphorus to the soil (Durán-Lara et al., 2020; Kakar et al., 2020) effect on the content of the element is no different from the GPR system. However, the ORP system uses bio-extract to add TK, but it is inferior to the fertilizer in the GRP system. Therefore, the TK content in soil may be

planted using for yield product (Atapattu et al., 2018), where the situation in TKR is the dough, and the high-temperature effect at the biodegradable microbial process in the soil cannot function effectively (Sarkar et al., 2017).

### Rice Production Quantities in ORP Systems in TKR

The quantity of rice production in ORP systems in TKR is 2093–2968 kg/ha for Jasmine rice, so organic rice yields about 2090–2544 kg/ha (Panpluem et al., 2019; Suwanmaneepong et al., 2020). On the other hand, the GRP yield in TKR is about 1255–1739 kg/ha, which is below the average rice yield in the country's northeast region average of 1810 kg/ha (Suebpongsang et al., 2020). The rice yield quantity in ORP has affected rice growing methods (Jierwiriyapant et al., 2012) and farming practices in ways that can be classified as follows:

1. ORP uses transplantation for rice growing; this practice has a greater effect on rice yield quantities than broadcasting or drum seeding (Dendup et al., 2018). GRP uses broadcasting most frequently for rice growing.
2. In the TKR zone, the average size of paddy fields in ORP systems is 0.57 ha; it is about 1.2 ha in GRP systems. Smaller fields allow farmers to better care for their crops.
3. ORP includes labor-intensive activities, such as weed and pest control.

## CONCLUSION

All indicators in ORP and GRP systems in the TKR zone are lower than the rate in soil that is suitable for rice production. In particular, the quantity of macroelements is TN > TK > TP at a ratio of 338:3:1. The quantity of TK in GRP is higher than in ORP, which is significant ($p < 0.05$). The value of SOM and SOC, including the C/N ratio, is not significant in either ORP or GRP, and the SOM/SOC ratio of 3.45 is higher than the reasonable rate of about 1.9. Soil improvement techniques in ORP systems—manure only and manure combined with green manure—have higher pH values ($p < 0.05$) than fertilizer only input, but the TK value in fields using manure only input and manure combined with green manure is lower ($p < 0.05$) than the fertilizer only input. Burning fields increases EC in the soil ($p < 0.05$), and the relationship of EC to % NaCl ($r = 0.741$) affects soil salinity levels. This study determined that ORP is a more effective system in the TKR zone because yields are impacted by farming practices different from the intensive farming methods used in GRP.

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## REFERENCES

Araragi, M., Motomura, S., Koyama, T., Matsuguchi, T., Chammek, C., Niamsrichand, N., Tangcham, B., Patiyuth, S., & Seirayosakol, A. (1978). Dynamic behavior of soil nitrogen in paddy field soils of Thailand. *JARQ*, *12*(2), 79-85.

Arunrat, N., Kongsurankan, P., Sereenonchai, S., & Hatano, R. (2020). Soil organic carbon in sandy paddy fields of northeast Thailand: A review. *Agronomy*, *10*(8), 1061. https://doi.org/10.3390/agronomy10081061

Atapattu, A. J., Rohtha-Prasantha, B. D., Amaratunga, K. S. P., & Marambe, B. (2018). Increased rate of potassium fertilizer at the time of heading enhances the quality of direct seeded rice. *Chemical and Biological Technologies in Agriculture*, *5*, 22. https://doi.org/10.1186/s40538-018-0136-x

Avasthe, R. K., Babu, S., Singh, R., & Das, S. K. (2018). Impact of organic food production on soil quality. In D. Anup, K. P. Mohapatra, S. V. Ngachan, A. S. Panwar, D. J. Rajkhowa, G. I. Ramkrushna, & L. Jayanta (Eds.), *Conservation agriculture for advancing food security in changing climate* (pp. 409-418). Today and Tomorrow's Printers and Publishers.

Bianchi, R. S., Miyazawa, M., de Oliveira, L. E., & Pavan, A. M. (2008). Relationship between the mass of organic matter and carbon in soil. *Brazilian Archives of Biology and Technology*, *51*(2), 263-269. https://doi.org/10.1590/S1516-89132008000200005

Bray, R. H., & Kurtz, L. T. (1945). Determination of total, organic, and available forms of phosphorus in soils. *Soil Science*, *59*(1), 39-46. https://doi.org/10.1097/00010694-194501000-00006

Chen, J., Gong, Y., Wang, S., Guan, B., Balkovic, J., & Kraxner, F. (2019). To burn or retain crop residues on croplands? An integrated analysis of crop residue management in China. *Science of The Total Environment*, *662*, 141-150. https://doi.org/10.1016/j.scitotenv.2019.01.150

Cruz-Paredes, C., Tajmel, D., & Rousk, J. (2021). Can moisture affect temperature dependences of microbial growth and respiration?. *Soil Biology and Biochemistry*, *156*, 108223. https://doi.org/10.1016/j.soilbio.2021.108223

Cynthia, M. K., Frey, D. S., & Grandy, S. A. (2016). Direct evidence for microbial-derived soil organic matter formation and its ecophysiological controls. *Nature Communications*, *7*, 13630. https://doi.org/10.1038/ncomms13630

Dendup, C., Chhogyel, N., & Ngawang. (2018). Effects of different planting methods on rice (*Oryza sativa* L.) crop performance and cost of production. *Bhutanese Journal of Agriculture*, *1*(1), 13-22.

Durán-Lara, E., Valderrama, A., & Marican, A. (2020). Natural organic compound for application in organic farming. *Agriculture*, *10*(2), 41. https://doi.org/10.3390/agriculture10020041

Freitas, V. H. (2000). *Soil management and conservation for small farms: Strategies and methods of introduction, technologies and* equipment. FAO. https://www.fao.org/3/bl032e/bl032e.pdf

Haque, M. M., Datta, J., Ahmed, T., Ehsanullah, M., Karim, N. M., Akter, M. S., Iqbal, A. M., Baazeem, A., Hadifa, A., Ahmed, S., & Sabagh, E. A. (2021). Organic amendments boost soil fertility and rice productivity and reduce methane emissions from paddy fields under sub-tropical conditions. *Sustainability*, *13*(6), 3103. https://doi.org/10.3390/su13063103

Herique, O., & Faysse, N. (2020). A large-scale public programme to promote organic rice farming in

Thailand: Building solid foundations to enable farmers to engage?. *Organic Agriculture*, *11*(3), 27-40. https://doi.org/10.1007/s13165-020-00320-4

Islam, M. A., Ferdous, G., Akter, A., Hossain, M. M., & Nandwani, D. (2017). Effect of organic, inorganic fertilizers and plant spacing on the growth and yield of cabbage. *Agriculture*, *7*(4), 31. https://doi.org/10.3390/agriculture7040031

Jenkinson, D. S., & Powlson, D. S. (1976). The effects of biocidal treatments on metabolism in soil—V: A method for measuring soil biomass. *Soil Biology and Biochemistry*, *8*(3), 209-213. https://doi.org/10.1016/0038-0717(76)90005-5

Jierwiriyapant, P., Liangphansakul, O., Chulaphun, W., & Pichaya-satrapongs, T. (2012). Factors affecting organic rice production adoption of farmers in northern Thailand. *Chiang Mai University Journal of Natural Sciences (Special Issue on Agricultural and Natural Resources)*, *11*(1), 327-333.

Kakar, K., Xuan, T. D., Noori, Z., Aryan, S., & Gulab, G. (2020). Effects of organic and inorganic fertilizer application on growth, yield, and grain quality of rice. *Agriculture*, *10*(11), 544. https://doi.org/10.3390/agriculture10110544

Khaki, D. B., Honarjoo, N., Davatgar, N., Jalalian, A., & Golsefidi, H. T. (2017). Assessment of two soil fertility indexes to evaluate paddy fiends for rice cultivation. *Sustainability*, *9*(8), 1299. https://doi.org/10.3390/su9081299

Klingenfuß, C., Roßkopf, N., Walter, J., Heller, C., & Zeitz. (2014). Soil organic matter to soil organic carbo ratios of peatland soil substrates. *Geoderma*, *235-236*, 410-417. https://doi.org/10.1016/j.geoderma.2014.07.010

Land Development Department. (2021). *Soil series of Kula Rong Hai*. http://iddindee.ldd.go.th/SoilSeries/K_1/16_Series_(Ki).pdf

Li, J., Lu, J., Li, X., Ren, T., Cong, R., & Zhou, L. (2014). Dynamics of potassium release and adsorption on rice straw residue. *PLOS One*, *9*(2), e90440. https://doi.org/10.1371/journal.pone.0090440

Lin, H., & Fukushima, Y. (2016). Rice cultivation methods and their sustainability aspects: Organic and conventional rice production in industrialized tropical monsoon Asia with a dual cropping system. *Sustainability*, *8*(6), 529. https://doi.org/10.3390/su8060529

Loeffler, E., Thompson, W. P., & Liengsakul, M. (1993). Geomorphological development of the Thung Kula Ronghai. In V. Thiramongkol & V. Pisutha-Arnond (Eds.), *Symposium on Geomorphology and Quaternary Geology of Thailand* (pp. 123-130). Chulalongkorn University.

Möller, K. (2015). Effects of anaerobic digestion on soil carbon and nitrogen turnover, N emissions, and soil biological activity. *Agronomy for Sustainable Development*, *35*, 1021–1041. https://doi.org/10.1007/s13593-015-0284-3

Office of Agricultural Economics. (2019). *Rice yearly production, area, production, production/area, classifying by district, yearly cropping 2018/2019*. OAE. http://www.oae.go.th/assets/portals/1/fileups/prcaidata/files/major%20rice%2061%20dit.pdf

Panpluem, N., Mustafa, A., Huang, X., Wang, S., & Yin, C. (2019). Measuring the technical efficiency of certified organic rice producing farms in Yasothon province: Northeast Thailand. *Sustainability*, *11*(24), 6974. https://doi.org/10.3390/su11246974

Pathma, J., & Sakthivel, N. (2012). Microbial diversity of vermicompost bacteria that exhibit useful agricultural traits and waste management potential. *SpringerPlus*, *1*, 26. https://doi.org/10.1186/2193-1801-1-26

Powlson, D., Hirsch, R. P., & Brookes, C. P. (2001). The role of soil microorganisms in soil organic matter conservation in the tropics. *Nutrient Cycling in Agroecosystems*, *61*, 41–51. https://doi.org/10.1023/A:1013338028454

Pribyl, W. D. (2010). A critical review of the conventional SOC to SOM conversion factor. *Geoderma*, *156*(3-4), 75-83. https://doi.org/10.1016/j.geoderma.2010.02.003

Rahman, M. N., Hangs, R., & Schoenau, J. (2020). Influence of soil temperature and moisture on micronutrient supply, plant uptake, and biomass yield of wheat, pea, and canola. *Journal of Plant Nutrition*, *43*(6), 823-833. https://doi.org/10.1080/01904167.2020.1711941

Rahmann, G. (2011). Biodiversity and organic farming: What do we know?. *Landbauforschung – vTI Agriculture and Forestry Research*, *3*(61), 189–208.

Reeve, J., Hoagland, L., Villalbl, J. J., Patrick, C., Amaya, A., Cambardella, C., Davis, D. R., & Kathleen, D. (2016). Chapter Six - Organic farming, soil health, and food quality: Considering possible links. *Advances in Agronomy*, *137*, 319-367. https://doi.org/10.1016/bs.agron.2015.12.003

Ross, S. M. (1993). Organic matter in tropical soils: Current conditions, concerns and prospects for conservation. *Progress in Physical Geography: Earth and Environment*, *17*(3), 265-305. https://doi.org/10.1177/030913339301700301

Saenya, J., Anusontpornperm, S., Thanachit, S., & Kheoruenromne, I. (2015). Potential of paddy soils for jasmine rice production in Si Sa Ket Province, Northeast Thailand. *Asian Journal of Crop Science*, *7*(1), 34-47. https://doi.org/10.3923/ajcs.2015.34.47

Saetung, W., & Trelo-ges, V. (2017). Study on some soil physical and chemical properties on the aromo of jasmine rice by geographic information system in Tung Kula Rong Hai area. *International Journal of Scientific and Engineering Research*, *8*(9), 1205-1209.

Sarkar, M. I. U., Islam, M. N., Jahan, A., Islam, A., & Biswas, J. C. (2017). Rice straw as a source of potassium for wetland rice cultivation. *Geology, Ecology, and Landscapes*, *1*(3), 184-189. https://doi.org/10.1080/24749508.2017.1361145

Secretariat of the Senate. (2001). *Report for consider: The problem of management in Thung Kula Ronghai*. https://www.senate.go.th/document/Ext2187/2187959_0002.PDF

Sompob, W. (1986). Salinization in Northeast Thailand. *Southeast Asian Studies*, *24*(2), 133-153.

Suebpongsang P., Ekasingh, B., & Cramb. R. (2020). Commercialisation of rice farming in Northeast Thailand. In R. Cramb (Ed.), *White gold: The commercialisation of rice farming in the Lower Mekong Basin* (pp. 39-68). Palgrave Macmillan. https://doi.org/10.1007/978-981-15-0998-8_2

Supriyadi, S., Purwanto, P., Sarijan, A., Mekiuw, Y., Ustiatik, R., & Prahesti, R. R. (2017). The assessment of soil quality at paddy fields in Merauke, Indonesia. *Bulgarian Journal of Agricultural Science*, *23*(3), 443–448.

Suwanmaneepong, S., Kerdsriserm, C., Lepcha, N., Cavite, H. J., & Llones, A. C. (2020). Cost and return analysis of organic and conventional rice production in Chachoensoa province, Thailand. *Organic Agriculture*, *10*, 369-378. https://doi.org/10.1007/s13165-020-00280-9

Swift, S. R. (1996). Chapter 35: Organic matter characterization. In D. L. Sparks, A. L. Page, P. A. Helmke, R. H. Loeppert, P. N. Soltanpour, M. A. Tabatabai, C. T. Johnston, & M. E. Sumner (Eds.), *Methods of soil analysis: Part 3 chemical methods, 5.3* (pp. 1011-1069). Soil Science Society of America, Inc. https://doi.org/10.2136/sssabookser5.3.c35

Tang, C., & Chen, D. (2017). Interaction between soil moisture and air temperature in the Mississippi

River Basin. *Journal of Water Resource and Protection*, *9*(10), 1119–1131. https://doi.org/10.4236/jwarp.2017.910073

Thuansri, Y., & Morathop, N. (2016). The network development of organic rice farmers in Uttardit province: Case study of Wangapee sub-district, Mueang district, Uttaradit province. *Lampang Rajabhat University Journal*, *5*(2), 116-132.

Thummahitsakul, S., Subsinsungnern, R., Treerassapanich, N., Kunsanprasit, N., Puttirat, L., Kroeksakul, P., & Silprasit, K. (2018). Pesticide and heavy metal contamination: Potential health risks of some vegetables and fruits from a local market and family farm in Ongkharak District of Nakhon Nayok Province, Thailand. *Pertanika Journal of Tropical Agricultural Science*, *41*(3), 987-1001.

Ueasangkomsate, P., Suthiwartnarueput, K., & Chaveesuk, R. (2018). Understanding competitive advantage of organic agriculture through the natural-resource-based view: Case studies of tree organic rice producer networks. *Thammasart Review*, *21*(2), 179-200.

United States Department of Agriculture. (2011). *Soil quality indicators*. USDA. https://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/health/assessment/?cid=stelprdb1237387

United States Environmental Protection Agency. (1996). *Method 3050B: Acid digestion of sediments, sludges, and soils*. https://www.epa.gov/sites/production/files/2015-06/documents/epa-3050b.pdf

Zhou, W., Teng-Fei, L., Chen, A., Westby, P., & Ren, W. J. (2014). Soil physicochemical and biological properties of paddy-upland rotation: A review. *The Scientific World Journal*, *2014*, 8563521. https://doi.org/10.1155/2014/856352

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# Effect of *Streptomyces* Inoculation on *Ipomoea aquatica* and *Pachyrhizus erosus* Grown Under Salinity and Low Water Irrigation Conditions

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## ABSTRACT

The distribution of salty areas and drought conditions caused by climate change can limit successful crop production. The co-occurrence of salinity and drought gives a unique challenge for plant growth-promoting bacteria (PGPB) in agricultural purposes. In this study, the effect of irrigation and salinity on the abilities of isolates of plant growth-promoting bacteria (*Streptomyces* sp. St1 and St8) to promote the growth of *Ipomoea aquatica* and *Pachyrhizus erosus* was investigated. Both plants were planted in pots with combinations of salinity (non-saline or saline soil), different irrigation levels, and different bacterial inoculations. The results showed that the salinity decreased the root dry weight of *I. aquatica* and decreased the shoot and root dry weight of *P. erosus*. Salinity also decreased the tuber formation and root efficiency of *P. erosus*. Low irrigation and bacterial species did not affect either plant's shoot or root growth. However, the chlorophyll content in the leaves of both plants decreased in the inoculated plants compared to the non-inoculated plants. Among the three factors in this study, salinity was the most influential factor, and irrigation was the least effective factor on plant growth for both parts. Soil salinity may concern plant growth-promoting bacteria, and salt-tolerant strains may be an interesting choice for use in combination with saline and low water conditions.

*Keywords*: Drought stress, economic crop, plant growth-promoting bacteria, salt stress, *Streptomyces*

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## INTRODUCTION

Using plant growth-promoting bacteria (PGPB) is a promising environmentally friendly method to increase the growth of several plants for both agricultural and environmental purposes. However, salinity and drought can affect the growth of both plants and bacteria. Chloride ions are toxic to bacteria via induction of acidification in the cytoplasm (Rivera-Araya et al., 2020). A lack of available water and exposure to a high concentration of salt results in bacterial cells encountering hyperosmotic stress. This stress decreases microbial growth and inhibits many essential cellular functions (Guan et al., 2017). Soil salinity causes decreases in crop growth and yield. The germination rate, shoot length, root length, and biomass of many plant species that have received saline wastewater decrease with an increase in the salinity (Calheiros et al., 2012). In addition, plants exposed to salinity led to an increased sodium ion ($Na^+$) content in the tissue and induced oxidation stress in the plant (A. Kumar et al., 2021). Soluble salt accumulation in the root zone may disrupt plant water uptake and essential nutrient absorption (Leogrande & Vitti, 2018). In addition, drought stress increased the oxidation stress, chloroplast damage, and destruction of chlorophyll in plants (Munné-Bosch et al., 2001).

Several semi-arid and arid areas in Asia encounter drought and salinity problems, and they are distributed in South Asia, Central Asia, and North Africa (Aryal et al., 2020; Kilroy, 2015). In Thailand, there are around 2.3 million hectares of salt-affected soil, and more than three-quarters of this is in the north-eastern part of the country (Somsri & Pongwichian, 2015). The slight to moderate levels of saline soil in these areas are normally used to cultivate many crops in Thailand, including rice (Somsri & Pongwichian, 2015). In addition to the problems of salt-affected soil, climate change induces prolonged drought, which is an important issue because this decreases agricultural productivity (Aryal et al., 2020; Marks, 2011). Salt and drought stress expose plants to osmotic stress, nutrient deficiency, and ion imbalance in soil (Hussian et al., 2018; Shankar & Evelin, 2019), which results in subsequent decreases in their productivity.

There are several mechanisms in PGPB that can stimulate plant growth under drought and salt stresses. For example, ACC deaminase production could decrease the ethylene level in plants, indole-3-acetic acid (IAA) production increases the root surface area, which subsequently increases the water and nutrient uptake, exopolysaccharide production increases the soil water holding capacity, and phosphate solubilizing activity increases the phosphate uptake in plants (Ilangumaran & Smith, 2017; Ojuederie et al., 2019). Several PGPB has been used to stimulate plant growth under salt or drought stresses (Ansari et al., 2019; Batool et al., 2020; Bharti et al., 2016).

Among several PGPB species, successful use of the bacteria in genus *Streptomyces* has been reported to promote crop growth under drought or salt stress conditions. For example, *Streptomyces* sp. isolate IT25, which can produce ACC deaminase, could prevent yield losses in

tomatoes cultivated under drought stress (Abbasi et al., 2020). Actinobacteria's cell-free extract produced phytohormones and siderophores and induced plant reactive oxygen species scavengers and osmoprotectants, improved corn growth under normal and drought conditions (Warrad et al., 2020). *Streptomyces* strain C-2012 could increase the chlorophyll and carotenoid levels and reduce the $Na^+$ content in wheat cultivars Zarin and Gonbad, and this helped alleviate the negative effect of salt stress (Akbari et al., 2020). Most research studies have focused on only one stress, either salt or drought, but when using PGPB to stimulate the growth of plants under a combination of stresses, there is little work. It would be interesting for cultivation in drought and saline areas. In addition, different physiologies of plants may respond to a combination of these stresses and the inoculant strain in different ways.

Thus, this study was carried out to investigate the effect of irrigation, salinity, and isolates of PGPB on their ability to promote the growth of *I. aquatica* and *P. erosus*. *Streptomyces* sp. St1 and *Streptomyces* sp. St8, the selected isolates, were PGPB with the ability to produce indole-3-acetic acid (IAA) and phosphate solubilization (Somtrakoon et al., 2019). *Ipomoea aquatica* and *P. erosus* were the selected plant species with different habitats. *Ipomoea aquatica* is an herbaceous plant and has been reported to survive in saline soil, while *P. erosus* is a tuber plant and can grow in several parts of Thailand. These results will be useful for selecting potential PGPB to be used as biofertilizers in agricultural areas facing drought and salt stress in the future.

## MATERIALS AND METHODS

### Preparation of Immobilized Cells + Spores of *Streptomyces* St1 and St8

*Streptomyces* sp. St1 and *Streptomyces* sp. St8 was isolated from soil planted with mango trees in Kosumphisai District, Maha Sarakham Province, and Kalasin Province, respectively, by A. Sangdee. The morphology of the colonies and spore chains of these bacteria are shown in Figure 1. The immobilization of both isolates were done according to the method described in Somtrakoon et al. (2021). Briefly, *Streptomyces* sp. St1 and St8 were cultured in a half formulation of potato dextrose agar (PDA) (Himedia, India, pH 5.2–5.3) for 16 days. Then, the cells + spore suspensions of *Streptomyces* sp. St1 and St8 were scrapped and transferred into 0.85 % sodium chloride (NaCl). Coconut husk was autoclaved at 121 ºC for 15 min before use. Then the autoclaved coconut husk was soaked in the cells + spore suspensions of *Streptomyces* sp. St1 and St8 for 3 h. The cell numbers of *Streptomyces* sp. St1 and St8 in the coconut husk after the immobilization process were counted by the spread plate method with a half formulation of potato dextrose agar. Initially, both bacterial isolates were around $10^4$ cell/g of coconut husk. Then, 7 g of coconut husk with immobilized cells of *Streptomyces* sp. St1 or St8 were used in the experimental pots—autoclaved coconut husk without cells of *Streptomyces* sp. St1 and St8 were used in the control pots.

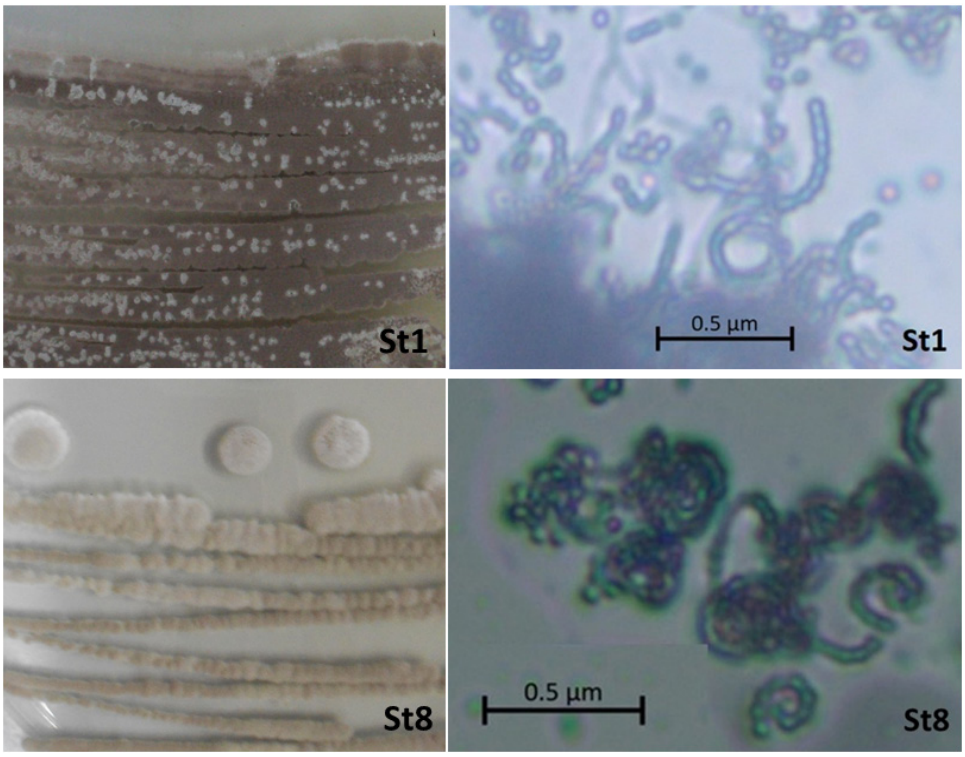


*Figure 1*. Morphology of colonies and spore chains of *Streptomyces* sp. St1 and *Streptomyces* sp. St8 growing on half formula of PDA for 14 days

## Soil Preparation and Experimental Design

The soil was collected from Takhianluan Sub-district, Muang District, Nakhon Sawan Province, Thailand, and sent for character analysis at the Central Laboratory (Thailand) Company Limited, Khonkaen Province, Thailand. Saline soil was prepared by adding 0.4 % w/w of NaCl to the soil before sending it for analysis. Soil without NaCl addition was used as the non-saline soil. The soil characteristics analyzed in this study were soil texture, pH, cation-exchange capacity, organic matter, available phosphorus, total nitrogen, and total potassium. The physical and chemical characteristics of these soils are listed in Table 1. The experiment was laid out in a 2x2x3 factorial completely randomized design (CRD). The details of each factor for each plant are shown in Table 2. Each treatment was performed in seven replicates.

## Stimulation of Growth of Crops Under Low Water Irrigation

According to a previous study, the pot experiment was done with some adaptation (Somtrakoon et al., 2022). The seeds of

*I. aquatica* and *P. erosus*, which were commercial seeds from Nakhon Ratchasima Province, Thailand, were soaked in distilled water for 5 h before sowing in each pot containing 2 kg soil/pot. After thinning the five-day-old, germinated seedlings to one plant per pot, the inoculation of immobilized bacteria in coconut husk was done. It was the first day of the experiment. The irrigation levels of *I. aquatica* and *P. erosus* were different. For *I. aquatica*, 20 mL of distilled water was watered every day in normal irrigation, and 20 mL of distilled water was used every other day in low irrigation. For *P. erosus*, 20 mL of distilled water was watered every other day in normal irrigation, and 20 mL of distilled water was used every other day in low irrigation. The experiment ended 45 days after germination for both plants—the total levels of *Streptomyces* sp. St1, St8, and other bacteria in the soil from each treatment were counted on a half formulation of PDA on the last day of the experiment. Each plant's shoot and root growth were determined, including length, dry weight, chlorophyll content, and leaf number. The chlorophyll content was determined according to the method described in Huang et al. (2004). Briefly, 200 mg of small leaves were incubated in 80% acetone at 4 ºC for 24 h in the dark. The absorbance of the acetone solution was measured with a spectrophotometer at 645 and 663 nm and the chlorophyll concentrations (mg/mL) were calculated using the following equations:

$$[\text{Chl } a] = [12.7 \times A663] - [2.69 \times A645]$$

$$[\text{Chl } b] = [22.9 \times A645] - [4.68 \times A663]$$

$$[\text{Total Chl}] = [8.02 \times A663] + [20.2 \times A645]$$

where,
Chl *a* = Chlorophyll *a* content
Chl *b* = Chlorophyll *b* content
Total Chl = Total chlorophyll content
A645 = Absorbance at a wavelength of 645 nm
A663 = Absorbance at a wavelength of 663 nm

### Statistical Analysis

One-way, two-way, and three-way analyses of variance tests were used for the main effects at $P \leq 0.05$. In addition, pairwise comparisons of mean treatment of parameters for the significant effect were carried out using the least square difference test (LSD test) at $P \leq 0.05$.

## RESULTS AND DISCUSSION

### Shoot and Root Growth of *Ipomoea aquatica*

Bacterial inoculation, salinity, and irrigation did not affect the shoot growth of *I. aquatica*. On the other hand, these factors affected the root growth of *I. aquatica* (Table 3). Salinity decreased the root dry weight significantly while low irrigation increased the root length of *I. aquatica*. Following inoculation with *Streptomyces* sp. St8, the root dry weight of *I. aquatica* in treatment 6 was increased compared to treatment 12.

Table 1

*Characteristics of soil used in this study*

| Characteristic | Non-saline soil | Saline soil | Method |
|---|---|---|---|
| Soil texture | Sandy loam | Sandy loam | Mechanical analysis, pipette method |
| % sand | 67.46 % | 65.27 % | |
| % silt | 22.76 % | 18.66 % | |
| % clay | 9.78 % | 16.07 % | |
| Electrical conductivity | 1.33 | 2.61 ds/m | A handbook of soil analysis (Chemical and physical method) 1/2553 |
| pH | 7.80 | 7.94 | A handbook of soil analysis (Chemical and physical method) 1/2553 |
| Organic matter | 0.13 % | 0.17 % | A handbook of soil analysis (Chemical and physical method) 1/2553 |
| Available phosphorus | 237.80 mg/kg | 243.43 mg/kg | A handbook of soil analysis (Chemical and physical method) 1/2553 |
| Total nitrogen | 0.20 % | 0.27 % | A handbook of soil analysis (Chemical and physical method) 1/2553 |
| Total potassium (Total $K_2O$) | 0.54 % | 0.54 % | Manual of fertilizer analysis, APSRDO, DOA; 4/2551 |

*Note*. Commercial analysis at Central Laboratory (Thailand) Company Limited, Khonkaen Province, Thailand

Table 2

*Details of each treatment in this experiment*

| Treatment no. | Factor 1soil | Factor 2 irrigation | Factor 3 bacterial isolates |
|---|---|---|---|
| 1 | Non-saline soil | Normal irrigation | Non-inoculation |
| 2 | | | *Streptomyces* sp. St1 |
| 3 | | | *Streptomyces* sp. St8 |
| 4 | | Low irrigation | Non-inoculation |
| 5 | | | *Streptomyces* sp. St1 |
| 6 | | | *Streptomyces* sp. St8 |

Table 2 *(Continue)*

| Treatment no. | Factor 1 soil | Factor 2 irrigation | Factor 3 bacterial isolates |
|---|---|---|---|
| 7 | Saline soil | Normal irrigation | Non-inoculation |
| 8 | | | *Streptomyces* sp. St1 |
| 9 | | | *Streptomyces* sp. St8 |
| 10 | | Low irrigation | Non-inoculation |
| 11 | | | *Streptomyces* sp. St1 |
| 12 | | | *Streptomyces* sp. St8 |

In addition, *Streptomyces* sp. St1 inoculation tended to decrease the root length of *I. aquatica* compared with the *Streptomyces* sp. St8 inoculation. *Streptomyces* sp. St8 inoculation to *I. aquatica* growing in treatment 3 decreased the root length, shorter than those growing in treatment 6 (Table 4).

Inoculation with *Streptomyces* sp. St1 and St8 tended to increase the specific root length of *I. aquatica* in treatments 8-9 and 11–12 compared with treatments 2–3 and 5–6. The root to shoot ratio of *I. aquatica* tended to increase in treatments 4 and 10, but the root to shoot ratio of *I. aquatica* inoculated with *Streptomyces* sp. St1 and St8 tended to increase in treatments 5–6 only, but not observed in treatments 11–12. This result showed that low irrigation to *I. aquatica* tended to decrease the root efficiency to produce shoot biomass in both soils. *Streptomyces* inoculation to *I. aquatica* receiving low irrigation could resemble the root efficiency of those receiving normal irrigation in saline soil, but it is still deceased in non-saline soil (Table 4).

All factors, salinity, irrigation, and bacterial inoculation affected the chlorophyll content in *I. aquatica* in several ways. Salinity significantly increased the chlorophyll content, while *Streptomyces* inoculation decreased. In addition, low irrigation decreased the leaf size (Figure 2) and the chlorophyll *a* and total chlorophyll contents significantly. However, when considered for each soil separately, the inoculation of *Streptomyces* sp. St8 to *I. aquatica* in treatment 6 increased the chlorophyll a and total chlorophyll contents, which were 2.40 and 3.90 mg/mL respectively, and 4.68 and 7.72 mg/mL respectively in treatment 12 when compared with *I. aquatica* in treatments 3 and 9 (1.86 and 2.92 mg/mL in non-saline soil and 2.24 and 6.49 mg/mL in saline soil, respectively), as shown in Table 5.

Decreases in length and biomass are often found in plants exposed to salt or drought stresses. Increased oxidation stress, chloroplast damage, and destruction of chlorophyll followed by the plant senescence process were observed to start (Munné-Bosch et al., 2001). Maintaining the chlorophyll content under salt stress

Table 3

*Effect of soil, irrigation, and bacterial isolate on* Ipomoea aquatica *growth traits*

| | Number of leaves | Shoot length (cm) | Shoot dry weight (g) | Root length (cm) | Root dry weight (g) | Chlorophyll *a* (mg/ml) | Chlorophyll *b* (mg/ml) | Total chlorophyll (mg/ml) |
|---|---|---|---|---|---|---|---|---|
| Soil (factor 1) | | | | | | | | |
| Non-saline soil | 3.0b | 18.0 | 0.04 | 5.5 | 0.022a | 3.22b | 1.91b | 5.13b |
| Saline soil | 3.9a | 16.0 | 0.03 | 4.9 | 0.013b | 3.25a | 4.27a | 7.53a |
| *F*-test | * | ns | ns | ns | ** | ** | ** | ** |
| Irrigation (factor 2) | | | | | | | | |
| Normal irrigation | 3.8 | 15.7 | 0.04 | 4.4b | 0.015 | 3.28a | 2.93b | 6.20b |
| Low irrigation | 3.2 | 18.2 | 0.03 | 6.0a | 0.020 | 3.19b | 3.26a | 6.45a |
| *F*-test | ns | ns | ns | ** | ns | ** | ** | ** |
| Bacterial isolate (factor 3) | | | | | | | | |
| Control | 3.5 | 17.3 | 0.03 | 4.9ab | 0.015 | 4.10a | 4.23a | 8.33a |
| St1 | 3.0 | 15.6 | 0.03 | 4.7b | 0.016 | 2.81b | 2.58b | 5.40b |
| St8 | 3.9 | 18.0 | 0.04 | 6.0a | 0.022 | 2.80b | 2.46b | 5.26c |
| *F*-test | ns | ns | ns | * | ns | ** | ** | ** |
| *F*-test | | | | | | | | |
| Soil x irrigation | ns | ns | ns | ns | ** | ** | ** | ** |
| Soil x bacterial isolate | ns | ns | ns | ns | ns | ** | ** | ** |
| Irrigation x bacterial isolate | ns | ns | ns | ns | ns | ** | ** | ** |
| Soil x irrigation x bacterial isolate | ns | ns | ns | * | ns | ** | ** | ** |

*Note*. Different lower-case letters show significant differences within each factor by LSD test at $P \leq 0.05$; Abbreviations: ns, *, ** denote non-significance ($P \geq 0.05$), statistical significance ($P \leq 0.05$), and high statistical significance ($P \leq 0.01$), respectively.

Table 4

*Growth of* Ipomoea aquatica *in presence or absence of* Streptomyces *sp. when cultivated under non-saline soil and saline conditions for 45 days (Mean ± Standard Error)*

| | | Shoot | | |
|---|---|---|---|---|
| | | Leaf number | Length (cm) | Dry weight (g) |
| **Non-inoculation** | | | | |
| Non-saline soil | Normal irrigation (T1) | 3.7 ± 0.98Aa | 16.7 ± 0.72Aa | 0.030 ± 0.003Aa |
| | Low irrigation (T4) | 2.8 ± 0.41Aa | 19.3 ± 2.28Aa | 0.042 ± 0.006Aa |
| Saline soil | Normal irrigation (T7) | 4.8 ± 0.74Aa | 18.1 ± 1.38Aa | 0.046 ± 0.008Aa |
| | Low irrigation (T10) | 3.0 ± 0.47Aa | 15.1 ± 2.79Aa | 0.162 ± 0.020Aa |
| ***Streptomyces* sp. St1** | | | | |
| Non-saline soil | Normal irrigation (T2) | 3.4 ± 0.46Aa | 18.5 ± 4.59Aa | 0.041 ± 0.011Aa |
| | Low irrigation (T5) | 2.0 ± 0.40Aa | 16.4 ± 4.41Aa | 0.036 ± 0.010Aa |
| Saline soil | Normal irrigation (T8) | 3.0 ± 0.61Aa | 11.0 ± 1.91Aa | 0.024 ± 0.004Aa |
| | Low irrigation (T11) | 3.8 ± 0.96Aa | 16.6 ± 1.06Aa | 0.173 ± 0.025Aa |
| ***Streptomyces* sp. St8** | | | | |
| Non-saline soil | Normal irrigation (T3) | 3.4 ± 0.46Aa | 13.4 ± 2.91Aa | 0.037 ± 0.010Aa |
| | Low irrigation (T6) | 3.0 ± 0.35Aa | 23.5 ± 0.93Aa | 0.043 ± 0.003Aa |
| Saline soil | Normal irrigation (T9) | 4.5 ± 0.75Aa | 16.8 ± 3.43Aa | 0.049 ± 0.011Aa |
| | Low irrigation (T12) | 4.7 ± 0.77Aa | 18.4 ± 1.85Aa | 0.204 ± 0.019Aa |

Table 4 *(Continue)*

| | | Root | | | |
|---|---|---|---|---|---|
| | | Length (cm) | Dry weight (g) | Specific root length (m/g) | Root to shoot ratio |
| **Non-inoculation** | | | | | |
| Non-saline soil | Normal irrigation (T1) | 5.3 ± 1.31Aa | 0.011 ± 0.003Aa | 5.02 | 0.358 |
| | Low irrigation (T4) | 4.9 ± 0.51Aa | 0.021 ± 0.004Aa | 2.36 | 0.498 |
| Saline soil | Normal irrigation (T7) | 4.2 ± 0.35Aa | 0.016 ± 0.004Aa | 2.55 | 0.354 |
| | Low irrigation (T10) | 5.3 ± 0.17Aa | 0.012 ± 0.004Aa | 4.52 | 0.604 |
| ***Streptomyces* sp. St1** | | | | | |
| Non-saline soil | Normal irrigation (T2) | 4.3 ± 0.78Aa | 0.016 ± 0.004Aa | 2.70 | 0.389 |
| | Low irrigation (T5) | 6.8 ± 0.79Aa | 0.030 ± 0.007Aa | 2.23 | 0.849 |
| Saline soil | Normal irrigation (T8) | 3.5 ± 0.28Aa | 0.009 ± 0.003Aa | 3.71 | 0.394 |
| | Low irrigation (T11) | 4.2 ± 0.72Aa | 0.010 ± 0.002Ba | 4.14 | 0.374 |
| ***Streptomyces* sp. St8** | | | | | |
| Non-saline soil | Normal irrigation (T3) | 3.4 ± 0.53Ab | 0.018 ± 0.005Aa | 1.90 | 0.478 |
| | Low irrigation (T6) | 8.6 ± 0.56Aa | 0.036 ± 0.003Aa | 2.38 | 0.838 |
| Saline soil | Normal irrigation (T9) | 5.7 ± 0.73Aa | 0.020 ± 0.003Aa | 2.82 | 0.410 |
| | Low irrigation (T12) | 6.4 ± 0.71Aa | 0.013 ± 0.002Ba | 5.08 | 0.390 |

*Note*. Different lower-case letters show significant differences between different irrigation treatments for the same soil at each bacterial inoculation by LSD test at $P \leq 0.05$; Different capital letters show significant differences between different soils for the same irrigation treatment at each bacterial inoculation by LSD test at $P \leq 0.05$

Table 5

*Chlorophyll content in leaves of* Ipomoea aquatica *and* Pachyrhizus erosus *in presence or absence of* Streptomyces *sp. when cultivated under non-saline soil and saline conditions for 45 days (Mean ± Standard Error)*

| | | ***Ipomoea aquatica*** | | |
|---|---|---|---|---|
| | | Chlorophyll *a* content (mg/ml) | Chlorophyll *b* content (mg/ml) | Total chlorophyll content (mg/ml) |
| **Non-Saline Soil** | | | | |
| Normal irrigation | Non-inoculation (T1) | 5.75 ± 0.01Aa | 3.34 ± 0.02Aa | 9.08 ± 0.02Aa |
| | *Streptomyces* sp. St1 (T2) | 3.51 ± 0.01Ab | 2.25 ± 0.01Ab | 5.77 ± 0.01Ab |
| | *Streptomyces* sp. St8 (T3) | 1.86 ± 0.01Bc | 1.06 ± 0.01Bc | 2.92 ± 0.01Bc |
| Low irrigation | Non-inoculation (T4) | 3.33 ± 0.01Ba | 1.74 ± 0.01Ba | 5.07 ± 0.01Ba |
| | *Streptomyces* sp. St1 (T5) | 2.44 ± 0.01Bb | 1.58 ± 0.01Bb | 4.03 ± 0.00Bb |
| | *Streptomyces* sp. St8 (T6) | 2.40 ± 0.01Ac | 1.50 ± 0.02Ac | 3.90 ± 0.00Ac |
| **Saline Soil** | | | | |
| Normal irrigation | Non-inoculation (T7) | 3.46 ± 0.03Ba | 4.69 ± 0.20Ba | 8.16 ± 0.18Ba |
| | *Streptomyces* sp. St1 (T8) | 2.86 ± 0.01Ab | 1.96 ± 0.03Bb | 4.82 ± 0.02Bc |
| | *Streptomyces* sp. St8 (T9) | 2.24 ± 0.01Bc | 4.25 ± 0.02Aa | 6.49 ± 0.01Bb |
| Low irrigation | Non-inoculation (T10) | 3.87 ± 0.01Ab | 7.16 ± 0.07Aa | 11.03 ± 0.05Aa |
| | *Streptomyces* sp. St1 (T11) | 2.43 ± 0.03Bc | 4.53 ± 0.18Ab | 6.96 ± 0.15Ac |
| | *Streptomyces* sp. St8 (T12) | 4.68 ± 0.01Aa | 3.04 ± 0.04Bc | 7.72 ± 0.03Ab |

Table 5 *(Continue)*

| | | *Pachyrhizus erosus* | | |
|---|---|---|---|---|
| | | Chlorophyll *a* content (mg/ml) | Chlorophyll *b* content (mg/ml) | Total chlorophyll content (mg/ml) |
| **Non-Saline Soil** | | | | |
| Normal irrigation | Non-inoculation (T1) | 3.21 ± 0.06A | 3.83 ± 0.35A | 7.04±0.28A |
| | *Streptomyces* sp. St1 (T2) | B.D. | B.D. | B.D. |
| | *Streptomyces* sp. St8 (T3) | B.D. | B.D. | B.D. |
| Low irrigation | Non-inoculation (T4) | 1.29 ± 0.13Ba | 1.77 ± 0.12Bb | 3.06 ± 0.07Bb |
| | *Streptomyces* sp. St1 (T5) | 1.88 ± 0.17a | 3.19 ± 0.33a | 5.07 ± 0.39a |
| | *Streptomyces* sp. St8 (T6) | 1.28 ± 0.13a | 2.05 ± 0.20b | 3.33 ± 0.33b |
| **Saline Soil** | | | | |
| Normal irrigation | Non-inoculation (T7) | 3.43 ± 0.01A | 3.87 ± 0.20A | 7.30 ± 0.19A |
| | *Streptomyces* sp. St1 (T8) | B.D. | B.D. | B.D. |
| | *Streptomyces* sp. St8 (T9) | B.D. | B.D. | B.D. |
| Low irrigation | Non-inoculation (T10) | 1.45 ± 0.29Ba | 2.04 ± 0.30Aa | 3.49 ± 0.59Ba |
| | *Streptomyces* sp. St1 (T11) | B.D. | B.D. | B.D. |
| | *Streptomyces* sp. St8 (T12) | 1.44 ± 0.34a | 1.38 ± 0.46a | 2.82 ± 0.19a |

*Note.* Different lower-case letters show significant differences between different inoculations for the same irrigation treatment at each soil by LSD test at $P \leq 0.05$; Different capital letters show significant differences between different irrigation treatments for the same inoculation at each soil by LSD test at $P \leq 0.05$; B.D. means that all leaves were brown and dry

indicated plant tolerance. The chlorophyll content decreased in gac (*Momordica cochinchinensis*) leaves related to an increase in the electrolyte leakage and antioxidant enzymes (Jumpa et al., 2017). Drought stress also decreased the total chlorophyll content in finger millet leaves, but inoculation with some drought-tolerant bacteria could increase the chlorophyll content (Chandra et al., 2018). However, only the root dry weight of *I. aquatica* was decreased by salinity, and only chlorophyll content was decreased by low irrigation when inoculation with *Streptomyces* sp. St1 or non-inoculation. Inoculation with *Streptomyces* sp. St8 seemed helpful for the root length and chlorophyll content of *I. aquatica* growing in low irrigation and non-saline soil.

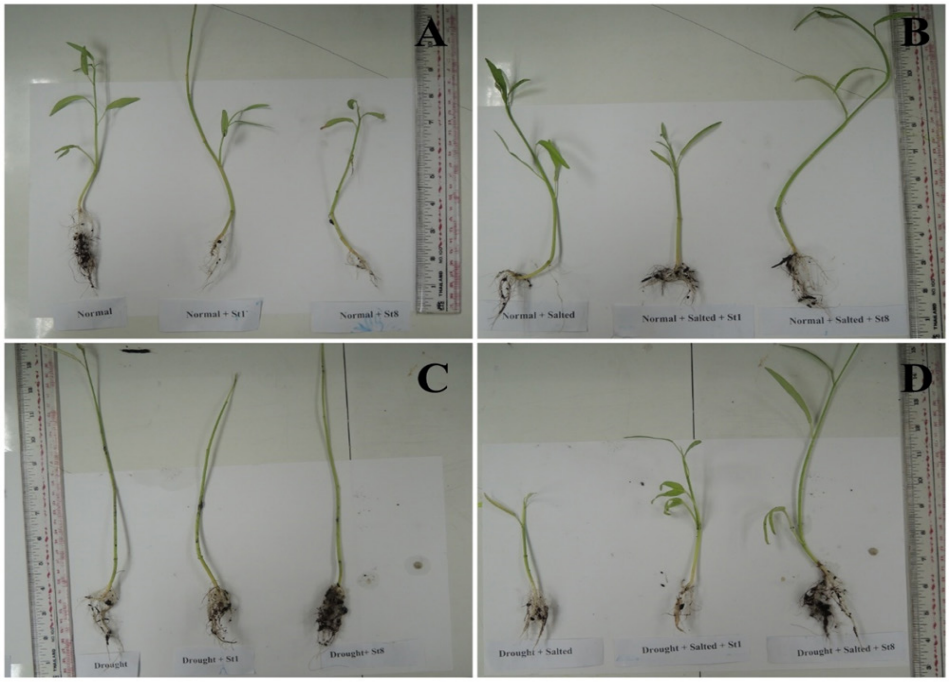


*Figure 2*. Characteristics of shoot and root of *Ipomoea aquatica* grown under non-saline soil + normal irrigation (A), saline soil + normal irrigation (B), non-saline soil + low water (C), and saline soil + low water conditions (D)

### Shoot and Root Growth of *Pachyrhizus erosus*

Only salinity decreased the shoot and root dry weight of *P. erosus* significantly. At the same time, irrigation and bacterial inoculation did not affect the shoot and root growth of *P. erosus* but affected the chlorophyll content in the plant (Table 6). The interaction of drought and salinity stress affected the leaf area and relative water in canola leaves (Sharif et al., 2018). An additive effect of water deficit and salinity

was found on the chlorophyll fluorescence in tomato leaves (Kautz et al., 2014). However, an interaction of soil salinity and irrigation was found clearly on the root dry weight and chlorophyll content in leaves of *I. aqutica*, but it was not seen for *P. erosus*. Only irrigation affected the chlorophyll content in the leaves of *P. erosus*.

Salinity decreased the dry shoot weight of *P. erosus* when receiving normal irrigation and inoculation with *Streptomyces* sp. St1 or non-inoculation. On the other hand, salinity decreased the root dry weight of *P. erosus* when receiving normal irrigation and non-inoculation only (Table 7). The specific root length of *P. erosus* tended to increase in saline soil compared with non-saline soil under all irrigation and bacterial inoculation treatments. For example, the specific root length of *P. erosus* growing in treatment 7 was 2.55 when it was 1.89 in treatment 1 (Table 7). The root to shoot ratio of *P. erosus* tended to decrease in treatments 10-12 (0.085–0.127) compared with that grown in treatments 7–9 (0.112–0.199). The result revealed that low irrigation to *P. erosus* in saline soil tended to increase the efficiency of the root to produce shoot biomass. Tuber formation of *P. erosus* decreased when planted in saline soil with normal irrigation and bacterial inoculation (Table 7).

The leaves of *P. erosus* in some *Streptomyces* inoculation treatments (all *Streptomyces* inoculations for normal irrigation in both soils and *Streptomyces* St8 for low irrigation in saline soil) turned yellow and white after day 30 of the experiment (Figure 3). On day 45 of the experiment, these white leaves turned brown and dry. The chlorophyll content was not measured for these treatments. Low irrigation decreased the chlorophyll content of *P. erosus* leaves, while salinity did not affect the chlorophyll in these leaves. For example, the total chlorophyll content in *P. erosus* leaves grown in treatment 1 was 7.04 mg/ml while they were 3.06–5.07 mg/mL for treatments 4-6. In addition, the total chlorophyll contents in the leaves of *P. erosus* grown in treatments 1 and 4–6 were 3.06–7.04 mg/mL while they were 2.82–7.30 mg/mL in treatments 7, 10, and 12 (Table 5). The chlorophyll content in the leaves of *P. erosus* significantly decreased when grown with low irrigation both in saline and non-saline soil. *Streptomyces* inoculation did not alleviate this effect on the chlorophyll content in *P. erosus* leaves.

Among these factors, salinity affected both plants' growth more than the other factors. Normally, the responses of plants to salinity and drought are similar, which are hyperosmotic and oxidative stress (Jumpa et al., 2017). However, salinity could enhance the $Na^+$ accumulation, disrupting plant cells ion homeostasis (A. Kumar et al., 2021). In addition, salinity did not decrease the plant health of *I. aquatica*. It may be due to the concentration of sodium chloride used in this study as it was in the range that *I. aquatica* could tolerate (Cha-um et al., 2007). The low irrigation in this experiment may not have stressed both plants enough. Generally, drought stress induces premature leaf senescence via reduced photosynthesis

Table 6

*Effect of soil, irrigation, and bacterial isolate on* Pachyrhizus erosus *growth traits*

| | Number of leaves | Shoot length (cm) | Shoot dry weight (g) | Root length (cm) | Root dry weight (g) | Chlorophyll *a* (mg/ml) | Chlorophyll *b* (mg/ml) | Total chlorophyll (mg/ml) |
|---|---|---|---|---|---|---|---|---|
| Soil (factor 1) | | | | | | | | |
| Non-saline soil | 3.0 | 46.2 | 0.20a | 5.2 | 0.024a | 1.92 | 2.71 | 4.63 |
| Saline soil | 2.7 | 44.3 | 0.15b | 4.8 | 0.015b | 2.11 | 2.43 | 4.54 |
| *F*-test | ns | ns | ** | ns | * | ns | ns | ns |
| Irrigation (factor 2) | | | | | | | | |
| Normal irrigation | 3.2 | 43.7 | 0.18 | 4.8 | 0.021 | 3.32a | 3.85a | 7.17a |
| Low irrigation | 2.5 | 46.8 | 0.17 | 5.2 | 0.017 | 1.47b | 2.09b | 3.56b |
| *F*-test | ns | ns | ns | ns | ns | ** | ** | ** |
| Bacterial isolate (factor 3) | | | | | | | | |
| Control | 2.8 | 44.4 | 0.16 | 5.2 | 0.023 | 2.35 | 2.88a | 5.22a |
| St1 | 3.3 | 44.1 | 0.19 | 5.0 | 0.018 | 1.88 | 3.19a | 5.07a |
| St8 | 2.5 | 47.2 | 0.17 | 4.8 | 0.017 | 1.36 | 1.72b | 3.08b |
| *F*-test | ns | ns | ns | ns | ns | ns | * | ** |
| *F*-test | | | | | | | | |
| Soil x irrigation | ns | ns | ns | ns | ns | ns | ns | ns |
| Soil x bacterial isolate | ns | ns | ns | ns | ns | ns | ns | ns |
| Irrigation x bacterial Isolate | ns | ns | ns | ns | ns | - | - | - |
| Soil x irrigation x Bacterial isolate | ns | ns | ns | ns | ns | - | - | - |

*Note.* Different lower-case letters show significant differences within each factor by LSD test at $P \leq 0.05$; Abbreviations: ns, *, ** denote non-significance ($P \geq 0.05$), statistical significance ($P \leq 0.05$), and high statistical significance ($P \leq 0.01$), respectively

Table 7

*Growth of* Pachyrhizus erosus *in presence or absence of* Streptomyces *sp. when cultivated under non-saline soil and saline conditions for 45 days (Mean ± Standard Error)*

| | | Shoot | | |
|---|---|---|---|---|
| | | Leaf number | Length (cm) | Dry weight (g) |
| **Normal irrigation** | | | | |
| Non-inoculation | Non-Saline Soil (T1) | 3.2 ± 0.52Aa | 48.7 ± 3.08Aa | 0.220 ± 0.016Aa |
| | Saline Soil (T7) | 3.2 ± 0.52Aa | 40.8 ± 4.00Aa | 0.161 ± 0.026Ab |
| *Streptomyces* sp. St1 | Non-Saline Soil (T2) | 3.8 ± 0.22Aa | 44.0 ± 4.14Aa | 0.226 ± 0.015Aa |
| | Saline Soil (T8) | 4.0 ± 0.28Aa | 44.2 ± 4.79Aa | 0.188 ± 0.017Ab |
| *Streptomyces* sp. St8 | Non-Saline Soil (T3) | 3.0 ± 0.61Aa | 45.9 ± 3.24Aa | 0.209 ± 0.042Aa |
| | Saline Soil (T9) | 2.0 ± 0.00Aa | 38.6 ± 4.63Aa | 0.086 ± 0.021Aa |
| **Low irrigation** | | | | |
| Non-inoculation | Non-Saline Soil (T4) | 2.7 ± 0.38Aa | 51.4 ± 4.09Aa | 0.162 ± 0.020Aa |
| | Saline Soil (T10) | 2.0 ± 0.71Aa | 36.6 ± 3.11Aa | 0.109 ± 0.037Aa |
| *Streptomyces* sp. St1 | Non-Saline Soil (T5) | 2.9 ± 0.24Aa | 40.8 ± 4.32Aa | 0.173 ± 0.025Aa |
| | Saline Soil (T11) | 2.5 ± 1.06Aa | 47.5 ± 8.84Aa | 0.188 ± 0.006Aa |
| *Streptomyces* sp. St8 | Non-Saline Soil (T6) | 2.6 ± 0.49Aa | 46.6 ± 2.86Aa | 0.204 ± 0.019Aa |
| | Saline Soil (T12) | 2.6 ± 0.36Aa | 58.0 ± 9.22Aa | 0.175 ± 0.018Aa |

Table 7 *(Continue)*

| | | Root | | | | |
|---|---|---|---|---|---|---|
| | | Length (cm) | Dry weight (g) | Specific root length (m/g) | Root to shoot ratio | % Tuber formation |
| **Normal irrigation** | | | | | | |
| Non-inoculation | Non-Saline Soil (T1) | 5.9 ± 0.26Aa | 0.031 ± 0.007Aa | 1.89 | 0.142 | 71.4 % |
| | Saline Soil (T7) | 4.5 ± 0 .39Aa | 0.018 ± 0.004Ab | 2.55 | 0.108 | 57.1 % |
| *Streptomyces* sp. St1 | Non-Saline Soil (T2) | 5.3 ± 0.12Aa | 0.022 ± 0.003Aa | 2.40 | 0.097 | 14.3 % |
| | Saline Soil (T8) | 4.7 ± 1.00Aa | 0.013 ± 0.002Aa | 3.65 | 0.069 | 28.6 % |
| *Streptomyces* sp. St8 | Non-Saline Soil (T3) | 3.9 ± 0.25Aa | 0.012 ± 0.001Aa | 3.40 | 0.055 | 14.3 % |
| | Saline Soil (T9) | 4.8 ± 1.91Aa | 0.008 ± 0.003Aa | 5.65 | 0.099 | 0.0 % |
| **Low irrigation** | | | | | | |
| Non-inoculation | Non-Saline Soil (T4) | 5.2 ± 0.25Aa | 0.032 ± 0.005Aa | 1.62 | 0.199 | 100 % |
| | Saline Soil (T10) | 5.2 ± 0.71Aa | 0.010 ± 0.001Aa | 5.25 | 0.091 | 0.0 % |
| *Streptomyces* sp. St1 | Non-Saline Soil (T5) | 6.1 ± 0.48Aa | 0.020 ± 0.007Aa | 3.14 | 0.112 | 57.1 % |
| | Saline Soil (T11) | 4.2 ± 0.11Aa | 0.016 ± 0.005Aa | 2.58 | 0.085 | 28.6 % |
| *Streptomyces* sp. St8 | Non-Saline Soil (T6) | 5.1 ± 0.24Aa | 0.027 ± 0.006Aa | 1.92 | 0.131 | 71.4 % |
| | Saline Soil (T12) | 5.3 ± 0.57Aa | 0.022 ± 0.003Aa | 2.40 | 0.127 | 28.6 % |

*Note.* Different lower-case letters show significant differences between different soils for the same inoculation at each irrigation treatment by LSD test at $P \leq 0.05$; Different capital letters show significant differences between different inoculations for the same soil at each irrigation treatment by LSD test at $P \leq 0.05$


*Figure 3*. Characteristics of shoot and root of *Pachyrhizus erosus* grown under non-saline soil + normal irrigation (A), saline soil + normal irrigation (B), non-saline soil + low irrigation (C), and saline soil + low irrigation (D)

and affects the membrane integrity (Ergo et al., 2021), leading to a decreasing leaf number, but the leaf numbers of both plants in this experiment were not affected by low irrigation.

Bacterial inoculation had negative effects on the chlorophyll content of both plants and only *Streptomyces* sp. St8 increased the root length of *I. aquatica*. Despite *Streptomyces* sp. St1 and St8 having been reported to produce IAA and solubilize phosphate at the laboratory scale (Somtrakoon et al., 2019), both activities of these bacterial isolates did not support the growth of *I. aquatica* and *P. erosus* in the pot experiment in this study. It might be due to several reasons, including the initial number of bacterial cells used being too low ($10^4$ cfu/g of coconut husk) and the low number of microbial inoculants that might not have the ability to compete with the indigenous bacteria in the soil. Colonies of both isolates were not detected after enumeration from the soil on half formulations of PDA from each treatment at the end of the experiment. The colonies of other bacteria overgrew the agar plates of half formulation PDA. Moreover, the organic matter, total nitrogen, and total potassium in the soil used in this study were low (Table 1), which may not favor the growth and survival of *Streptomyces* sp. St1 and St8 after introduction to the soil. *Streptomyces* sp. St1 and St8 could not be adapted to growth under low water irrigation

or saline soil in this study. Indigenous bacteria isolated from drought or saline soils have been suggested as a source for biofertilizers (B. L. Kumar & Gopal, 2015).

Normally, plant growth-promoting bacteria used under salt stress should be tolerant to salt stress—for example, inoculation of *Pseudomonas* sp. Strain UW4, wildtype or mutant OxtreS that tolerated 0.2 M NaCl could protect tomato plant growth from salt stress when irrigated with 0.2 M NaCl (Orozco-Mosqueda et al., 2019). In the laboratory, *Streptomyces* sp. St1 and St8 could conserve their phosphate solubilization and IAA production abilities when exposed to NaCl. Within 35 days, the IAA production of *Streptomyces* sp. St1 in PDA + 3.4% NaCl did not decrease while the phosphate solubilization decreased 9% in PDA + 2.55% NaCl compared with those grown on PDA without NaCl. In addition, IAA production by *Streptomyces* sp. St8 in PDA + 1.7 % NaCl decreased 9%, and phosphate solubilization decreased 39% in PDA + 4.25% NaCl compared with those grown on PDA without NaCl (Pukmak et al., 2020), but both isolates did not enhance plant growth when introduced to the soil. In summary, the salinity of the soil might be more of a concern for PGPB used under a combination of drought and salinity. Developing *Streptomyces* sp. St1 and St8 as biofertilizers might not be appropriate because the plant growth-promoting activities of both bacterial isolates did not boost and promote the growth of the tested plants.

## CONCLUSION

Salinity affected the success of plant growth-promoting bacteria used in *Ipomoea aquatica* and *Pachyrhizus erosus* cropping more than the water-limited effect. Based on the shoot and root growth, there were significant interactions between salinity and irrigation on root dry weight of *I. aquatica* only. All factors had significant interactions with the chlorophyll content of *I. aquatica*. Salinity was the most effective factor, and irrigation was the least influential factor on both plants' growth. The importance of considering the plant growth-promoting bacterial strain for use under salt and drought conditions is the salt tolerance of these bacteria.

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## REFERENCES

Abbasi, S., Sadeghi, A., & Safaie, N. (2020). *Streptomyces* alleviate drought stress in tomato plants and modulate the expression of transcription factors ERF1 and WRKY70 genes. *Scientia Horticulturae*, *265*, 109206. https://doi.org/10.1016/j.scienta.2020.109206

Akbari, A., Gharanjik, S., Koobaz, P., & Sadeghi, A. (2020). Plant growth-promoting *Streptomyces* strains are selectively interacting with the wheat cultivars especially in saline conditions. *Heliyon*, *6*(2), e03445. https://doi.org/10.1016/j.heliyon.2020.e03445

Ansari, M., Shekari, F., Mohammadi, M.H., Juhos, K., Végvári, G., & Biró, B. (2019). Salt-tolerant plant growth-promoting bacteria enhanced salinity tolerance of salt-tolerant alfalfa (*Medicago sativa* L.) cultivars at high salinity. *Acta Physiologiae Plantarum*, *41*, 195. https://doi.org/10.1007/s11738-019-2988-5

Aryal, J. P., Sapkota, T. B., Khurana, R., Khatri-chhetri, A., Rahut, D. B., & Jat, M. L. (2020). Climate change and agriculture in South Asia: Adaptation options in smallholder production systems. *Environment, Development and Sustainability*, *22*, 5045–5075. https://doi.org/10.1007/s10668-019-00414-4

Batool, T., Ali, S., Seleiman, M. F., Naveed, N. H., Ali, A., Ahmed, K., Abid, M., Rizwan, M., Shahid, M. R., Alotaibi, M., Al-Ashkar, I., & Mubushar, M. (2020). Plant growth-promoting rhizobacteria alleviates drought stress in potato in response to suppressive oxidative stress and antioxidant enzymes activities. *Scientific Reports*, *10*, 16975. https://doi.org/10.1038/s41598-020-73489-z

Bharti, N., Pandey, S. S., Barnawal, D., Patel, V. K., & Kalra, A. (2016). Plant growth-promoting rhizobacteria *Dietzia natronolimnaea* modulates the expression of stress responsive genes providing protection of wheat from salinity stress. *Scientific Reports*, *6*, 34768. https://doi.org/10.1038/srep34768

Calheiros, C. S. C., Silva, G., Quitério, P. V. B., Crispim, L. F. C., Brix, H., Moura, S. C., & Castro, P. M. L. (2012). Toxicity of high salinity tannery wastewater and effects on constructed wetland plants. *International Journal of Phytoremediation*, *14*(7), 669-680. https://doi.org/10.1080/15226514.2011.619233

Chandra, D., Srivastava, R., Glick, B. R., & Sharma A. K. (2018). Drought-tolerant *Pseudomonas* spp. improve the growth performance of finger millet (*Eleusine coracana* (L.) Gaertn.) under non-stressed and drought-stressed conditions. *Pedosphere*, *28*(2), 227-240. https://doi.org/10.1016/S1002-0160(18)60013-X

Cha-um, S. Roytrakul, S., Kirdmanee, C., Akutagawa, I., & Takagaki, M. (2007). A rapid method for identifying salt tolerant water convolvulus (*Ipomoea aquatica* Forsk) under in vitro photoautotrophic conditions. *Plant Stress*, *1*(2), 228-234.

Ergo, V. V., Veas, R. E., Vega, C. R. C., Lascano, R., & Carrera, C. S. (2021). Leaf photosynthesis and senescence in heated and droughted field-grown soybean with contrasting seed protein concentration. *Plant Physiology and Biochemistry*, *166*, 437-447. https://doi.org/10.1016/j.plaphy.2021.06.008

Guan, N., Jianghua Li, J., Shin, H., Du, G., Chen, J., & Liu, L. (2017). Microbial response to environmental stresses: From fundamental mechanisms to practical applications. *Applied Microbiology and Biotechnology*, *101*, 3991–4008. https://doi.org/10.1007/s00253-017-8264-y

Huang, X.-D., El-Alawi, Y., Penrose, D. M., Glick, B. R., & Greenberg, B. M. (2004). Response of three grass species to creosote during phytoremediation. *Environmental Pollution*, *130*(3), 453-363. https://doi.org/10.1016/j.envpol.2003.12.018

Hussian, H., Hussain, S., Khaliq, A., Ashraf, U., Anjum, S. A, Men, S., & Wang, L. (2018). Chilling and drought stresses in crop plants: implications, cross talk, and potential management opportunities. *Frontiers in Plant Science*, *9*, 393. https://doi.org/10.3389/fpls.2018.00393

IIangumaran, G., & Smith, D. L. (2017). Plant growth-promoting rhizobacteria in amelioration of salinity stress: A systems biology perspective. *Frontiers in Plant Science*, *8*, 1768. https://doi.org/10.3389/fpls.2017.01768

Jumpa, T., Pattanagul, W., & Songsri, P. (2017). Effects of salinity stress on some physiological traits in gac (*Momordica cochinchinensis* (Lour.)

Spreng.). *Khon Kaen Agriculture Journal*, *45*(suppl.1), 255-260.

Kautz, B., Noga, G., & Hunsche, M. (2014). Sensing drought- and salinity-imposed stresses on tomato leaves by means of fluorescence techniques. *Plant Growth Regulation*, *73*, 279–288. https://doi.org/10.1007/s10725-014-9888-x

Kilroy, G. (2015). A review of the biophysical impacts of climate change in three hotspot regions in Africa and Asia. *Regional Environmental Change*, *15*, 771-782. https://doi.org/10.1007/s10113-014-0709-6

Kumar B. L., & Gopal, D. V. R. S. (2015). Effective role of indigenous microorganisms for sustainable environment. *3 Biotech*, *5*, 867–876. https://doi.org/10.1007/s13205-015-0293-6

Kumar, A., Mann, A., Kumar, A., Kumar, N., & Meena, B. L. (2021). Physiological response of diverse halophytes to high salinity through ionic accumulation and ROS scavenging. *International Journal of Phytoremediation*, *23*(10), 1041-1051. https://doi.org/10.1080/15226514.2021.1874289

Leogrande, R., & Vitti, C. (2018). Use of organic amendments to reclaim saline and sodic soils: A review. *Arid Land Research and Management*, *33*(1), 1-21. https://doi.org/10.1080/15324982.2018.1498038

Marks, D. (2011). Climate change and Thailand: Impact and response. *Contemporary Southeast Asia*, *33*(2), 229-258. https://doi.org/10.1355/cs33-2d

Munné-Bosch, S., Jubany-Marí, T., & Alegre, L. (2001). Drought-induced senescence is characterized by a loss of antioxidant defences in chloroplasts. *Plant Cell and Environment*, *24*(12), 1319-1327. https://doi.org/10.1046/j.1365-3040.2001.00794.x

Ojuederie, O. B., Olanrewaju, O. S., & Babalola, O. O. (2019). Plant growth-promoting rhizobacterial mitigation of drought stress in crop plants: Implications for sustainable agriculture. *Agronomy*, *9*(11), 712. https://doi.org/10.3390/agronomy9110712

Orozco-Mosqueda, M. C., Duan, J., DiBernardo, M., Zetter, E., Campos-Garcia, J., Glick, B. R., & Santoyo, G. (2019). The production of ACC deaminase and trehalose by the plant growth-promoting bacterium *Pseudomonas* sp. UW4 synergistically protect tomato plants against salt stress. *Frontiers in Microbiology*, *10*, 1392. https://doi.org/10.3389/fmicb.2019.01392

Pukmak, S., Somtrakoon, K., Saengdee, A., Chouychai, W., & Khompan, W. (2020, February 12). *Effect of sodium chloride on indole -3-acetic acid production and phosphate solubilization of plant growth-promoting bacteria* [Paper presentation]. Proceeding of the 6th Pibulsongkram Research 2020, Phitsanulok, Thailand. https://research.psru.ac.th/PBR2020/files/PBR2020_FullSciences.pdf

Rivera-Araya, J., Huynh, N. D., Kaszuba, R., Chávez, R., Schlömann, M., & Levicán, G. (2020). Mechanisms of NaCl-tolerance in acidophilic iron-oxidizing bacteria and archaea: Comparative genomic predictions and insights. *Hydrometallurgy*, *194*, 105334. https://doi.org/10.1016/j.hydromet.2020.105334

Shankar, V., & Evelin, H. (2019). Strategies for reclamation of saline soils. In B. Giri & A. Varma (Eds.), *Microorganisms in saline environments: Strategies and functions* (Vol. 56, pp. 439-449). Springer. https://doi.org/10.1007/978-3-030-18975-4_19

Sharif, P., Seyedsalehi, M., Paladino, O., Van Damme, P., Sillanpää, M., & Sharifi, A. A. (2018). Effect of drought and salinity stresses on morphological and physiological characteristics of canola. *International Journal of Environmental Science and Technology*, *15*, 1859–1866. https://doi.org/10.1007/s13762-017-1508-7

Somsri, A., & Pongwichian, P. (2015). Salt-affected soils and management in Thailand. *Bulletin of the Society of Sea Water Science, Japan*, *69*(5), 319-325. https://doi.org/10.11457/swsj.69.319

Somtrakoon, K., Sangdee, A., & Chouychai, W. (2019). Roles of plant growth-promoting bacteria on growth of ornamental plants grown in anthracene-spiked soil. *Journal of Agricultural Research and Extension*, *36*(2), 11-21.

Somtrakoon, K., Sangdee, A., & Chouychai, W. (2021). Effect of *Streptomyces* sp. St1 on growth of and potential to stimulate anthracene removal by sunn hemp (*Crotalaria juncea*) grown in anthracene-contaminated soil. *Songklanakarin Journal of Science and Technology*, *43*(3), 615-622.

Somtrakoon, K., Sangdee, A., & Chouychai, W. (2022). Maintaining growth of aquatic morning glory under drought condition by *Paenibacillus* sp. $BSR_{1\text{-}1}$. *Trends in Science. 19*(5), 2884. https://doi.org/10.48048/tis.2022.2884

Warrad, M., Hassan, Y. M., Mohamed, M. S. M., Hagagy, N., Al-Maghrabi, O. A., Selim, S., Saleh, A. M., & AbdElgawad, H. (2020). A bioactive fraction from *Streptomyces* sp. enhances maize tolerance against drought stress. *Journal of Microbiology and Biotechnology*, *30*(8), 1156-1168. https://doi.org/10.4014/jmb.2003.03034

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# Suitable Materials for *Paenibacillus* sp. $BSR_{1-1}$ Immobilization and Crop Growth Stimulation under Low Water Condition

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## ABSTRACT

Agricultural challenges due to a water shortage are factors limiting plant growth and productivity worldwide. One way to improve plant growth under unsuitable conditions is to use plant growth-promoting bacteria (PGPB). The objective of this study was to investigate the ability of PGPB to increase peanut, rice, and sweet corn growth under low water conditions. Suitable agricultural materials were selected first to be used in *Paenibacillus* sp. $BSR_{1-1}$ immobilization. The materials were water hyacinth, reed, and coconut husk. Water hyacinth maintained the bacterial cell number when kept at either -4, 4, or 27-30 °C for both storage times, and water hyacinth soaked with a bacterial cell suspension prepared in 0.5 % ammonium sulfate ($(NH_4)_2SO_4$) + 1 % glucose was the most suitable method to immobilize the bacterial cells. *Paenibacillus* sp. $BSR_{1-1}$ with indole-3-acetic acid (IAA) and exopolysaccharide-producing abilities significantly increased root growth of peanuts under the low water condition. Root length and dry weight of inoculated peanut grown under low water conditions were 138.91 % and 156.51 % higher than uninoculated peanut, respectively. This bacterial isolate significantly increased rice shoot dry weight and root length under low and full water conditions. However, it only increased shoot length and root dry weight under the full water condition. *Paenibacillus* sp. $BSR_{1-1}$ increased the dry

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weight of sweet corn under both conditions but only increased the root length of sweet corn under the full water condition. The shoot dry weight of inoculated sweet corn under the low water condition was 170.59 % higher than that of the un-inoculated sweet corn. When rice received *Paenibacillus* sp. $BSR_{1-1}$ under the full water condition, and when peanuts received these bacteria under both conditions, they could produce more tillers and pods than the un-inoculated plants. Thus, *Paenibacillus* sp. $BSR_{1-1}$ was an appropriate strain to use as a biofertilizer for agricultural proposes in water-limited areas.

*Keywords*: Corn, low water, *Paenibacillus*, peanut, plant growth-promoting bacteria, rice

## INTRODUCTION

Drought exerts negative impacts on plant growth and yield in several ways, including decreasing the water-soluble nutrient diffusion to the plant root inducing oxidative stress in plants, which results in lipid peroxidation, membrane degradation, and protein degradation (Vurukonda et al., 2016). There are several suggestions to mitigate the adverse effects of drought on plant growth and yield, including using water-saving irrigation, short-cycle, and drought-tolerant plants, traditional breeding, and drought-tolerant transgenic plants (Food and Agriculture Organization of the United Nations [FAO], n.d.; Niu et al., 2018). Moreover, the application of PGPB is another means to stimulate the growth of plants under limited water (Niu et al., 2018). Important characteristics of PGPB to support plant growth include nitrogen fixation, phosphate solubilization, ACC deaminase activity, siderophore production, and plant growth regulator production (de Souza et al., 2015). There have been several research reports on the successful use of PGPB under low water conditions. For example, inoculation of *Zea mays* seed with exopolysaccharide-producing bacteria (*Pseudomonas aeruginosa* (Pa2)) could increase protein and sugar concentrations and decrease the activity of antioxidant enzymes in leaves under stress conditions (Naseem & Bano, 2014). Inoculations of lettuce (*Lactuca sativa*) with *Bacillus megaterium* TV 6D (B1) and *Bacillus subtilis* TV 12H (B2) significantly increased the plant growth, yield, and nutrient content grown under lower irrigation levels (Sahin et al., 2015). Also, the seed germination and seedling growth of foxtail millet (*Setaria italica*) inoculated with *Pseudomonas fluorescens* DR7, which could produce ACC deaminase and exopolysaccharide, were increased under drought stress because the moisture increased in inoculated soil (Niu et al., 2018). Moreover, inoculation of peanut shoots with *Bradyrhizobium* strain ESA 123 increased the number of nodules and activation of metabolic gene expression for plant protection under water deficit stress (Brito et al., 2019).

*Paenibacillus* sp. $BSR_{1-1}$ with the ability to produce IAA (Somtrakoon et al., 2019), ammonia, exopolysaccharide, and drought tolerance has been reported to stimulate the root growth of aquatic morning glory in our

previous study (Somtrakoon et al., 2022), which was used as a model of PGPB in this study. However, successful use of PGPB depends on their survival ability, as they need to compete with indigenous bacteria and settle around the root zone (de Souza et al., 2015). Using immobilized microbial cells is expected to overcome the limiting factors that restrict the use of PGPB in agricultural soil. Several advantages of using immobilized microbial cells have been reported, including maintaining high microbial biomass, preserving high microbial activity, the resistance of microbial cells to toxic chemicals, and providing long cellular viability (Bashan, 1998; Martins et al., 2013; Santos et al., 2019).

Several immobilization materials, including agar, sodium alginate, hydrogel, and composite materials, have been used as immobilized cell carriers for biochemical production and wastewater treatment (Lu et al., 2020; Martins et al., 2013). The possible mechanisms of immobilization technologies include adsorption onto the surface of immobilized materials, encapsulation in immobilized materials, entrapment within immobilization materials, and containment within a polymer (Lu et al., 2020). Important criteria for immobilized materials include insoluble, non-toxic, high stability, high diffusivity, easy immobilization process, high biomass retention, and cheap (Martins et al., 2013). Based on these suitable criteria for immobilized materials, agricultural residues can be used as carriers for inoculating microorganisms into agricultural soil. The benefits of carriers from agricultural residues are that they are environmentally friendly, easy to apply, provide high porosity, provide a high surface area for cell attachment and nutrient transfer (Kirdponpattara et al., 2021; Santos et al., 2019). Examples of natural carriers from agricultural residues include water hyacinth (Kirdponpattara et al., 2021), coconut husk, sawdust, and rice straw (Somtrakoon et al., 2022). The objectives of this study were to find suitable natural carriers for immobilization of *Paenibacillus* sp. $BSR_{1-1}$ and to investigate the ability of *Paenibacillus* sp. $BSR_{1-1}$ to stimulate the growth of peanut cultivar 'Tainan 9' (*Arachis hypogaea*), sweet corn (*Zea mays* var. saccharata), and rice cultivar 'KDML 105' (*Oryza sativa*) when cultivated under full and low water conditions.

## MATERIALS AND METHODS

### Plant Growth-Promoting Activity

*Paenibacillus* sp. $BSR_{1-1}$ was previously isolated from a paddy field in Wapi Pathum District, Maha Sarakham Province, Thailand, by Assoc. Prof. Aphidech Sangdee. It had 97 % similarity to *Paenibacillus polymyxa* based on a 16s rDNA sequence. These bacteria were cultured in nutrient agar and a 24 h culture of *Paenibacillus s*p. $BSR_{1-1}$ was used as an inoculum to test the promoting plant growth. Further plant growth-promoting activities were tested in this study, including ACC deaminase production, siderophore production, and potassium solubilization. ACC deaminase activity was screened by the method described in Penrose and Glick (2003). Potassium solubilization activity was

tested according to the method described in Prajapati and Modi (2012). Siderophore production was tested according to the methods described in Pérez-Miranda et al. (2007). Finally, carboxymethyl cellulose degradation was tested by the methods described in George et al. (2001).

**Suitable Preparation of Immobilized Cells in Agricultural Residues**

Agricultural residues, including coconut husk, reed, and water hyacinth, were cut into 1x1 cm pieces and autoclaved at 121 ºC for 15 min. Immobilized *Paenibacillus* sp. $BSR_{1-1}$ cells in agricultural residues were prepared by soaking these agricultural residues with a cell suspension of *Paenibacillus* sp. $BSR_{1-1}$ prepared in 0.85 % sodium chloride (NaCl) for 3 h. Cells of *Paenibacillus* sp. $BSR_{1-1}$ immobilized in each agricultural residue was kept at -4 ºC, 4 ºC, and 27-30 ºC for 10 and 30 days. *Paenibacillus* sp. $BSR_{1-1}$ cells were counted after being kept immobilized for 10 and 30 days (Table 1).

The suitable ammonium sulfate and glucose concentrations for immobilized *Paenibacillus* sp. $BSR_{1-1}$ in each agricultural residue were tested. Each agricultural residue was soaked in a cell suspension of *Paenibacillus* sp. and prepared with three formulations of ammonium sulfate and glucose (0.5 % ammonium sulfate + 1 % glucose, 1 % ammonium sulfate + 2 % glucose, and 1.5 % ammonium sulfate + 3 % glucose) for 3 h. The initial number of *Paenibacillus* sp. $BSR_{1-1}$ in each agricultural residue was counted after the immobilization process (Table 2). Then, the cells of *Paenibacillus* sp. $BSR_{1-1}$ immobilized in each agricultural residue was kept at 4 ºC for 10 and 30 days. The number of *Paenibacillus* sp. $BSR_{1-1}$ has counted again on days 10 and 30 after preparation. The best formulation of ammonium sulfate and glucose for maintaining cells of *Paenibacillus* sp. $BSR_{1-1}$ was sent to analyze the carbon and nitrogen ratio at the Central Laboratory (Thailand) Company Limited, Khonkaen Province.

**Pot Experiment**

Water hyacinth was soaked in a cell suspension of *Paenibacillus* sp. $BSR_{1-1}$ prepared in 0.5 % ammonium sulfate + 1 % glucose to prepare immobilized cells. Then, the immobilized cells of *Paenibacillus* sp. $BSR_{1-1}$ were used to stimulate the growth of peanut, rice, and sweet corn in a pot experiment. Seeds of peanut cultivar 'Tainan 9', rice cultivar 'KDML105', and sweet corn were received from a farmer in Chiangmai Sub-District, Pho-Chai District, Roi-Et Province, Thailand.

Soil from Donnong Village, Kham Riang Sub-District, Kantharawichai District, Maha Sarakham Province, Thailand, was collected for use in this study. The soil characteristics, including pH, organic matter, soil texture, available phosphorus, exchangeable potassium, exchangeable calcium, and exchangeable magnesium, at the beginning and the end of the experiment were determined via analysis at Soil-Fertilizer-Environment Scientific Development Project, Department of Soil

Science, Faculty of Agriculture, Kasetsart University, Thailand. The soil used in the pot experiment was prepared by autoclaving and divided into pots for planting the peanut, rice, and sweet corn. Soil moisture contents on days 1–4 after soaking the soil at room temperature were 13.07 ± 0.23 %, 8.84 ± 0.29 %, 7.66 ± 0.73 %, and 2.67 ± 0.79 %, respectively. The experimental pots for each plant were laid out in a completely randomized design with one factor. There were four treatments in this study: 1) uninoculated control at low water, 2) uninoculated control at full water, 3) inoculation of immobilized *Paenibacillus* sp. $BSR_{1-1}$ at low water, and 4) inoculation of immobilized *Paenibacillus* sp. $BSR_{1-1}$ at full water. Each treatment was performed as ten replicates. There were some differences between plant species, as described below.

**Peanut.** A total of 1.5 kg of the autoclaved soil was poured into each 24.13 cm diameter pot. Peanut seeds were submerged in distilled water for 48 h. Then, five germinated peanut seeds were added to each experimental pot. After seedling emergence, only one seedling of 12-day-old peanut with comparable sizes in each pot was left to grow. Then, 10 g of water hyacinth with immobilized cells of *Paenibacillus* sp. $BSR_{1-1}$ was spread on the surface of the soil on day 30. The initial concentration of *Paenibacillus* sp. $BSR_{1-1}$ immobilized in water hyacinth was 7.76 ± 0.34 log cfu/g. Free cells of *Paenibacillus* sp. $BSR_{1-1}$ with an initial concentration of 7.22 ± 0.06 and 7.88 ± 0.07 log cfu/ml were re-inoculated onto water hyacinth in the experiment pot on days 62 and 92, respectively. The irrigation of peanuts was divided into three phases. Firstly, water with 30 ml of water every day until day 30 of the experiment. The second phase started after the first inoculation of *Paenibacillus* sp. $BSR_{1-1}$ immobilized in water hyacinth and watered with 50 ml of water to each experimental pot every day for full water and every four days under the low water condition. The third phase of irrigation began when the peanut was 50 days old, and the irrigation of the low water peanut was changed to every other day until the end of the experiment. After flowering, more soil was poured around each peanut shoot in each pot experiment.

**Rice.** An amount of 1.25 kg of the autoclaved soil was poured into each experimental pot with 27.94 cm diameter. Rice seeds were immersed in distilled water for 48 h and transferred to the experimental pots, with each pot containing 15 seeds. After 12 days, the rice seedlings were thinned to 10 seedlings in each experimental pot. For the first 30 days of the experiment, the pots were watered every day. After that, water was poured into the rice pots under full water conditions until the water level was 5 cm above the soil surface. After that, rice planted under the low water condition was watered with 100 ml of water every four days. On day 50 of the experiment, only the low water pot was changed to 100 ml of water every day. Then, 10 g of water hyacinth with immobilized cells of *Paenibacillus* sp. $BSR_{1-1}$ was spread on the

soil surface on day 35 of the experiment under full and low water conditions. The initial cells of *Paenibacillus* sp. were at 7.76 ± 0.34 log cfu/g. Then, 15 ml of free cells of *Paenibacillus* sp. (7.22 ± 0.06 log cfu/ml) were re-inoculation onto water hyacinth in the experimental pots on day 62.

**Sweet Corn.** Sweet corn seeds were soaked in distilled water for 48 h. Then, five emerged seeds were inoculation into each experimental pot with a diameter of 15.24 cm containing 750 g of soil and thinned to one plant per pot on day 12. On day 30, 5 g of water hyacinth with immobilized cells of *Paenibacillus* sp. $BSR_{1-1}$ was spread on the soil surface. The initial cell number of *Paenibacillus* sp. $BSR_{1-1}$ in water hyacinth was 7.76 ± 0.34 log cfu/g. Each pot was watered every day until day 30 of the experiment. After that, 20 ml of water was poured into the experimental pots every day for the full water condition, and the schedule of watering was four days under the low water condition. After sweet corn was 50 days old, the irrigation pattern for the low water condition was changed to every other day until the end of the experiment. No chemical fertilizer was applied to the experimental pots because of only the effect of *Paenibacillus* sp. $BSR_{1-1}$ on the plant's growth was investigated. At the end of the experiment, 107-day-old peanuts, 78-day-old rice, and 65-day-old sweet corn were collected to analyze the plant growth parameters (root length, shoot length, number of leaves, shoot and root dry weight, and chlorophyll content in leaves). Chlorophyll content measurement was done according to Huang et al. (2004) for all plant leaves. Two pots of rice in low water condition when receiving *Paenibacillus* sp. $BSR_{1-1}$ were left until 100 days old to observe tiller and grain production.

### Statistical Analysis

Data in Tables 1, 2, 3, and 4 are expressed as mean ± standard error (SE). A one-way analysis of variance (ANOVA) was used for plant growth analysis, and two-way ANOVA was used for variance analysis for bacterial survival. The least-square difference (LSD) was used for the pairwise comparison of all experiments.

## RESULTS AND DISCUSSION

The plant growth-promoting bacteria used in this study, *Paenibacillus* sp. $BSR_{1-1}$, showed several abilities, such as IAA, exopolysaccharide and ammonia production, drought tolerance (Somtrakoon et al., 2019, 2022), and carboxy methyl cellulose degradation. However, this bacterial isolate could not solubilize phosphate (Somtrakoon et al., 2019) and potassium, and it could not produce siderophores and ACC deaminase. Our previous study indicated that the cells of *Paenibacillus* sp. $BSR_{1-1}$ immobilized in sawdust, rice straw, and coconut husk could induce aquatic morning glory root growth under drought conditions (Somtrakoon et al., 2022). Thus, this study was undertaken to determine more suitable agricultural materials and a suitable ratio for ammonium sulfate and glucose when preparing cell suspensions of *Paenibacillus* sp. $BSR_{1-1}$ for immobilized microbial cells. Coconut

husk has been used in a previous study (Somtrakoon et al., 2022) that also tested together with other agricultural residues, including reed and water hyacinth.

The results revealed that the most suitable agricultural residue to immobilize *Paenibacillus* sp. $BSR_{1-1}$ was water hyacinth. The results in Table 1 indicate that water hyacinth could maintain the cell number of *Paenibacillus* sp. $BSR_{1-1}$ when kept at either -4, 4, or 27–30 ºC. The cell numbers of *Paenibacillus* sp. $BSR_{1-1}$ immobilized in water hyacinth were not significantly different when kept at different temperatures (-4, 4, or 27–30 ºC) and storage times (10 and 30 days). The number of *Paenibacillus* sp. $BSR_{1-1}$ cells on day 30 were 7.61–9.02, 6.69–6.87, and 9.15–9.34 log cfu/g when immobilized in the reed, coconut husk, and water hyacinth, respectively. Moreover, 0.5% ammonium sulfate and 1% glucose were the suitable concentrations of the nutrients for preparing cell suspensions of *Paenibacillus* sp. $BSR_{1-1}$ immobilized in the reed, coconut husk, and water hyacinth. After storage for 30 days at 4 ºC, the cell numbers of *Paenibacillus* sp. $BSR_{1-1}$ immobilized in water hyacinth were 7.85 log cfu/g, which was significantly higher than that immobilized in reed (6.83 log cfu/g) and coconut husk (5.47 log cfu/g) (Table 2).

Thus, water hyacinth with 0.5% ammonium sulfate and 1% glucose was used as the agricultural material and solution to prepare the cell suspension of *Paenibacillus* sp. $BSR_{1-1}$ for the pot experiment. Based on the results from Table 2, the significant difference in cell number for each agricultural material at the beginning (Day 0) might depend on the sorption capacity of each agricultural material for the bacterial cells. A major factor that limited the successful use of microbial inoculants was a low cell number and low bacterial activity after introducing free cells to soil with biotic and abiotic stress in the environment (Partovinia & Rasekh, 2018). Thus, cell immobilization in water hyacinth was used to carry the cells of *Paenibacillus* sp. $BSR_{1-1}$ to the planted soil in this study. Immobilizing microbial cells in agricultural residues was expected to protect the microbial cells from environmental stress, thereby increasing microbial cell stability and density (Kirdponpattara et al., 2021). The main characteristic of the suitable carrier should be nontoxic to microbial cells (Yao et al., 2011). This study revealed that water hyacinth was the most suitable agricultural residue for immobilization of *Paenibacillus* sp. $BSR_{1-1}$ cells due to this material being able to maintain the microbial cell number at all storage temperatures. The aerenchyma tissue in water hyacinth has high porosity and a high ability to absorb water, which is useful for nutrient transfer and cell adsorption (Kirdponpattara et al., 2021). Moreover, the characteristics of the carrier surface may affect the microbial absorption onto them. A study by Kirdponpattara et al. (2021) reported that water hyacinth could immobilize yeast cells more than cocoon because the yeast cell had a high affinity to the water hyacinth surface than the other. Microbial cell absorption on carriers with smooth surfaces is difficult (Kirdponpattara et al., 2021).

Table 1
*Effect of storage temperature on cell numbers of* Paenibacillus *sp. $BSR_{1-1}$ immobilized in each agricultural material*

| Immobilized materials | - 4 ºC | 4 ºC | 27 - 30 ºC |
|---|---|---|---|
| Day 10 | | | |
| Water hyacinth | 9.27±0.036aA | 9.35±0.004aA | 9.13±0.044aA |
| Reed | 8.63±0.029bA | 8.18±0.088bA | 8.27±0.110bA |
| Coconut husk | 6.02±0.020cA | 6.67±0.104cA | 6.69±0.078cA |
| Material | ** | | |
| Temperature | ns | | |
| Material x Temperature | ** | | |
| Day 30 | | | |
| Water hyacinth | 9.34±0.018aA | 9.15±0.157aA | 9.16±0.038aA |
| Reed | 8.57±0.410bA | 9.02±0.276aA | 7.61±0.230bB |
| Coconut husk | 6.78±0.094cA | 6.87±0.176bA | 6.69±0.106cA |
| Material | ** | | |
| Temperature | * | | |
| Material x Temperature | * | | |

*Note*. Different lower-case letters show significant differences between agricultural residues for the same temperature, and different capital letters show significant differences between temperatures for the same agricultural residues. Symbols: ns, *, ** denote non-significance ($P$>0.05), statistical significance ($P$<0.05), and high statistical significance ($P$<0.01), respectively. The number of bacterial cells suspended at the beginning was approximate $10^{8}$–$10^{9}$ cfu/ ml (optical density of bacterial suspension at a wavelength of 600 nm = 0.5)

The carbon and nitrogen ratio of water hyacinth used in this study was 104.38: 1. The carbon and nitrogen ratio of water hyacinth after soaking in the cell suspension of *Paenibacillus* sp. $BSR_{1-1}$ was prepared in 0.5% ammonium sulfate, and 1% glucose was 57.08: 1. In general, a carbon and nitrogen ratio of less than 20 has a chance to degrade nitrogen (Truong & Marschner, 2018). Meanwhile, nitrogen immobilization could occur at a C: N ratio of more than 20 (Truong & Marschner, 2018), and the values ranged between 20–30, indicating the suitability of this material for compost production (Wu et al., 2017). Thus, the water hyacinth used in this study with a carbon and nitrogen ratio greater than 30 is suitable as it is difficult to degrade after application to the soil as a bacterial cell carrier. The addition of glucose and ammonium sulfate to water hyacinth did not change the carbon and nitrogen ratio, so it was optimum for composting. Thus, reuse of water hyacinth may be possible. Moreover, immobilized cells of *Paenibacillus* sp. $BSR_{1-1}$ can be stored at room temperature, and this is convenient when used in a real situation.

## Growth of Economic Crops Under Low Water Condition

While immobilized *Paenibacillus* sp. $BSR_{1-1}$ was inoculated in soil, the dry shoot weight, dry root weight, and root length of rice KDML105 and sweet corn grown under both full and low water conditions were higher than that grown in soil without *Paenibacillus* sp. $BSR_{1-1}$ inoculation. The shoot dry weight, root dry weight, and

Table 2
*Effect of glucose and ammonium sulfate concentration on cell numbers of* Paenibacillus *sp. $BSR_{1-1}$ in each immobilized material while kept at 4 ºC*

| Immobilized materials | 1% glucose + 0.5 % ammonium sulfate | 2% glucose + 1.0% ammonium sulfate | 3% glucose + 1.5% ammonium sulfate |
|---|---|---|---|
| Day 0 | | | |
| Water hyacinth | 9.06±0.05aA | 5.76±0.12bC | 6.21±0.04aB |
| Reed | 6.33±0.05bB | 7.24±0.19aA | 6.14±0.10aB |
| Coconut husk | 5.57±0.02cB | 5.43±0.08cB | 6.14±0.10aA |
| Material | ** | | |
| Nutrient | ** | | |
| Material x Nutrient | ** | | |
| Day 10 | | | |
| Water hyacinth | 8.59±0.40aA | 6.27±0.17bB | 6.53±0.23aB |
| Reed | 6.50±0.41bB | 7.69±0.11aA | 6.37±0.04aB |
| Coconut husk | 5.60±0.10bA | 5.37±0.05cA | 6.05±0.19aA |
| Material | ** | | |
| Nutrient | * | | |
| Material x Nutrient | ** | | |
| Day 30 | | | |
| Water hyacinth | 7.85±0.11aA | 5.59±0.13bB | 5.93±0.04bB |
| Reed | 6.83±0.08bAB | 7.09±0.35aA | 6.56±0.02aB |
| Coconut husk | 5.47±0.04cA | 5.48±0.17bA | 5.91±0.02bA |
| Material | ** | | |
| Nutrient | ** | | |
| Material x Nutrient | ** | | |

*Note*. Different lower-case letters show significant differences between agricultural residues for the same nutrient formulation, and different capital letters show significant differences between nutrients for the same agricultural residues. Symbols: * and ** denote statistical significance ($P$<0.05) and highly statistical significance ($P$<0.01), respectively. The number of bacterial cells suspended at the beginning was approximate $10^8$ - $10^9$ cfu/ ml (optical density of bacterial suspension at a wavelength of 600 nm = 0.5)

root length of rice grown in the presence of *Paenibacillus* sp. $BSR_{1-1}$ in soil under both conditions were around 0.15–0.17 g, 0.05–0.07 g, and 16.4–17.4 cm, respectively. Meanwhile, the shoot dry weight, root dry weight, and root length of rice grown in the absence of *Paenibacillus* sp. $BSR_{1-1}$ in soil under both conditions were around 0.04–0.10 g, 0.03–0.03 g, and 8.3–9.0 cm, respectively. The shoot dry weight, root dry weight, and root length of sweet corn grown in the presence of *Paenibacillus* sp. $BSR_{1-1}$ in soil under both conditions were around 0.58–0.63 g, 0.17–0.21 g, and 26.75–34.33 cm, respectively. The shoot dry weight, root dry weight, and root length of sweet corn grown in the absence of *Paenibacillus* sp. $BSR_{1-1}$ in soil under both conditions were around 0.30–0.34 g, 0.08 - 0.09 g, and 20.06–20.29 cm, respectively (Table 3). Soil inoculated with immobilized *Paenibacillus* sp. $BSR_{1-1}$ also stimulated the growth of peanuts. The shoot length of peanut grown under low water conditions was shorter (28.42–30.72 cm) than that grown under full water conditions (39.42–44.49 cm);

however, the inoculation of *Paenibacillus* sp. $BSR_{1-1}$ increased the root dry weight and root length of peanuts grown under full and low water conditions. The root dry weight and root length of peanut grown in the presence of *Paenibacillus* sp. $BSR_{1-1}$ in soil under both conditions were around 0.25–0.36 g and 23.4–28.2 cm, respectively. Meanwhile, the root dry weight and root length of peanut grown without *Paenibacillus* sp. $BSR_{1-1}$ inoculation under both conditions were only 0.09–0.23 g and 16.3–20.3 cm, respectively (Table 3). Inoculation of *Paenibacillus* sp. $BSR_{1-1}$ immobilized in water hyacinth to soil could stimulate peanut to produce pods. However, the peanut pods have grown without *Paenibacillus* sp. $BSR_{1-1}$ were absent (Table 4). The reason for this is not known.

*Paenibacillus* sp. $BSR_{1-1}$ could stimulate the growth of peanut, rice, and sweet corn, and these crops also responded to the low water condition in different ways. In this study, only the peanut grown under low water conditions had a higher root dry weight when grown under full water conditions. The root dry weight of peanut grown under low water condition with *Paenibacillus* sp. $BSR_{1-1}$ was 0.36 g, while the root dry weight of peanut grown under full water condition with *Paenibacillus* sp. $BSR_{1-1}$ was only 0.25 g. In addition, the root dry weight of peanuts grown under low water conditions without *Paenibacillus* sp. $BSR_{1-1}$ was 0.23 g, while the root dry weight of peanut grown under full water condition without *Paenibacillus* sp. $BSR_{1-1}$ was only 0.09 g. However, the root dry weight of rice and sweet corn is grown under low, and full water conditions were not significantly different. The inoculation of *Paenibacillus* sp. $BSR_{1-1}$ increased the root efficiency to produce shoot biomass of rice under the low water condition when considering the root-to-shoot ratio. The inoculation of *Paenibacillus* sp. $BSR_{1-1}$ increased the specific root length of rice under low water conditions while decreasing the specific root length of corn under both conditions. On the other hand, the inoculation of *Paenibacillus* sp. $BSR_{1-1}$ decreased the specific root length of peanuts under both conditions. There has been a report that high root growth under drought is usually found to increase water absorption (Farooq et al., 2009).

The number of peanut leaves planted under low water conditions was lower than those planted under full water conditions. Decreasing the leaf number is a mechanism for plants to decrease their water loss by transpiration. It is an adaptation for plants grown under drought conditions (Mohr & Schopfer, 1995). Meanwhile, the number of leaves in sweet corn planted under low and full water conditions were similar. Inoculation of *Paenibacillus* sp. $BSR_{1-1}$ to the soil planted with sweet corn under both conditions could increase the leaf number compared to that planted in the absence of *Paenibacillus* sp. $BSR_{1-1}$ (Table 3). Peanut and sweet corn responded to low water conditions in different ways. It may be due to the photosynthesis system of peanut and sweet corn, which were different. Peanuts are C3 plants, while sweet corn is a C4 plant. In general, the photorespiration rate of C3 plants is higher than in C4 plants (Mohr &

Table 3
*Shoot and root growth and chlorophyll content in peanut, rice, and sweet corn leaves under full and low water conditions with and without immobilized* Paenibacillus *sp. $BSR_{1-1}$ inoculation*

| | Leaf number | Shoot length (cm) | Shoot dry weight (g) | Root length (cm) | Root dry weight (g) | Specific root length (m/g) | Root to shoot ratio | Chlorophyll a (mg/ml) | Chlorophyll b (mg/ml) | Total chlorophyll (mg/ml) |
|---|---|---|---|---|---|---|---|---|---|---|
| Peanut | | | | | | | | | | |
| Low water + WH | 7.0±0.6 | 28.42±1.2b | 0.99±0.05b | 20.3±1.14b | 0.23±0.021b | 0.87 | 0.23 | 0.38±0.03a | 0.59±0.06a | 0.97±0.09a |
| Full water + WH | 10.6±0.7 | 44.49± 2.2a | 0.72±0.05c | 16.3±1.54b | 0.09±0.008c | 1.79 | 0.13 | 0.22±0.01b | 0.27±0.01b | 0.49±0.01b |
| Low water + $BSR_{1-1}$ | 7.7±0.3 | 30.72±3.4b | 1.09±0.06b | 28.2±2.34a | 0.36±0.039a | 0.79 | 0.33 | 0.34±0.02a | 0.54±0.02a | 0.88±0.04a |
| Full water + $BSR_{1-1}$ | 10.6±0.8 | 39.42±2.3a | 1.37±0.15a | 23.4±1.65ab | 0.25±0.015b | 0.93 | 0.18 | 0.18±0.01b | 0.31±0.04b | 0.49±0.05b |
| Rice | | | | | | | | | | |
| Low water + WH | 5.1±0.1 | 26.0±2.16a | 0.10±0.015b | 9.0±1.22b | 0.03±0.005b | 3.15 | 0.28 | 13.9±2.88b | 8.3±1.87b | 22.2±4.74b |
| Full water + WH | 4.7±0.1 | 17.6±0.99b | 0.04±0.006c | 8.3±0.72b | 0.03±0.003b | 2.79 | 0.65 | 23.5±0.09a | 26.1±2.32a | 49.6±2.40a |
| Low water + $BSR_{1-1}$ | 5.0±0.1 | 29.6±0.33a | 0.17±0.024a | 17.4±2.79a | 0.05±0.008b | 3.24 | 0.32 | 13.8±0.45b | 7.8±0.82b | 21.5±1.26b |
| Full water + $BSR_{1-1}$ | 4.8±0.1 | 28.8±1.28a | 0.15±0.012a | 16.4±1.35a | 0.07±0.009a | 2.42 | 0.45 | 23.4±0.11a | 27.7±3.68a | 51.2±3.66a |
| Sweet corn | | | | | | | | | | |
| Low water + WH | 3.1±0.2 | 24.70±2.36a | 0.34±0.03b | 20.29±3.25b | 0.08±0.02b | 2.40 | 0.24 | 10.35±0.21a | 4.90±0.09a | 15.24±0.12a |
| Full water + WH | 3.4±0.2 | 30.82±3.43a | 0.30±0.03b | 20.06±2.68b | 0.09±0.01b | 2.36 | 0.28 | 4.50±0.01c | 2.23±0.04c | 6.73±0.03c |
| Low water + $BSR_{1-1}$ | 4.1±0.3 | 27.03±1.86a | 0.58±0.06a | 26.75±1.81ab | 0.21±0.02a | 1.29 | 0.35 | 8.10±0.05b | 4.18±0.33b | 12.28±0.31b |
| Full water + $BSR_{1-1}$ | 4.6±0.2 | 26.87±2.44a | 0.63±0.07a | 34.33±3.82a | 0.17±0.02a | 1.97 | 0.28 | 3.47±0.03d | 1.25±0.02d | 4.71±0.02d |

*Note.* Different lower-case letters show significant differences between treatments for each plant ($P<0.05$). Abbreviations: WH = Water hyacinth; $BSR_{1-1}$ = *Paenibacillus* sp. $BSR_{1-1}$

Schopfer, 1995). Thus, C4 plants, including sweet corn in this study, can tolerate drought greater than peanut, which has a constant high photosynthetic rate.

The leaf number of rice, a C3 plant, grown under the low water condition was similar to that grown under the full condition either inoculated with or without *Paenibacillus* sp. $BSR_{1-1}$. The rice growth was not affected by the low water condition in this study. It may be that the rice growth was not reduced with low water conditions in this study. Rice can adapt to grow under low water conditions and can survive well. In addition, it was found that *Paenibacillus* sp. $BSR_{1-1}$ could promote rice growth under both low and full water conditions. The rice is grown in the presence of *Paenibacillus* sp. $BSR_{1-1}$ was better than that grown in the absence of *Paenibacillus* sp. $BSR_{1-1}$. Based on Table 3, the rice growth may be stimulated by *Paenibacillus* sp. $BSR_{1-1}$ with the greatest extent compared to the other plants. Inoculation of *Paenibacillus* sp. $BSR_{1-1}$ increased the survival of rice under both full and low water conditions; survival of rice grown under full water conditions was 77% and 34% when the soil was inoculated with and without *Paenibacillus* sp. $BSR_{1-1}$. Also, survival rates of 66% and 55% for rice found under low water condition inoculation with and without *Paenibacillus* sp. $BSR_{1-1}$ and rice are grown under the low water condition produced tillers and grain that were not observed in rice that did not receive *Paenibacillus* sp. $BSR_{1-1}$. *Paenibacillus* sp. $BSR_{1-1}$ could stimulate rice growth to the greatest extent compared to other plants because *Paenibacillus* sp. $BSR_{1-1}$ was isolated from soil in a paddy field. Thus, *Paenibacillus* sp. $BSR_{1-1}$ may be familiar and can enhance the soil planted with rice more than soil planted with other crops. The advantage of using indigenous bacteria includes the ability to adapt to the environment after introducing the bacteria into the environment again (Kumar & Gopal, 2015). Other plant growth-promoting bacteria have been reported to stimulate rice growth under drought stress. For example, bacterial inoculation of *Bacillus* sp. EN121, EN108, and EN43 increased biomass accumulation and grain yield of *Oryza sativa* L. variety MTU1010 growth under drought stress (Joshi et al., 2020). Bacterial inoculation of *Pseudomonas jessenii* R62 and *Pseudomonas synxantha* R81 also increased growth and stress-related enzymes in *Oryza sativa* L. varieties swarna and swarna sub1 grown under drought conditions (Gusain et al., 2014).

*Paenibacillus* sp. $BSR_{1-1}$ also stimulated the growth of peanut and sweet corn. The ability of *Paenibacillus* sp. $BSR_{1-1}$ stimulates the growth of plants comes from its plant growth-promoting activities, including exopolysaccharide, IAA, and ammonia production. The exopolysaccharides produced by bacteria could maintain soil water and increase water and nutrient uptake of plant roots from the soil and then promote plant growth under drought conditions (Vurukonda et al., 2016). Exopolysaccharide-producing bacteria could promote plant growth under drought conditions. For example, *Planomicrobium chinense* strain P1 and *Bacillus cereus* strain P2 stimulated the growth of wheat

and promoted drought tolerance in wheat. Exopolysaccharides released from bacteria act as a rhizosheath and can protect the plant root from drought for a long time (Khan & Bano, 2019). Moreover, foliar application of exopolysaccharides from *Pantoea alhagi* NX-1 increased drought tolerance in rice seedlings. Fresh weight and relative water content in rice were increased in the presence of exopolysaccharides (Sun et al., 2020). Moreover, exopolysaccharide-producing bacteria have been reported to decrease the rice exposure to toxic ions under high salt conditions using hydroxyl and carboxyl groups in the exopolysaccharide to bind and chelate sodium ions (Shultana et al., 2020a, 2020b). This mechanism may protect plants under drought conditions, which accumulate high concentrations of ions due to the low water content in the soil.

Other roles of *Paenibacillus* sp. $BSR_{1-1}$ stimulate the growth of plants is via IAA production. The possible important role of IAA producing bacteria in increasing plant growth under drought conditions is to modify the plant root architecture to increase the root tip number and root surface. These characteristics increase soil water and nutrient uptake (Ojuederie et al., 2019). However, an excessive amount of IAA can stimulate the transcription of genes that encode ACC synthase. This enzyme synthesizes ethylene precursors (1-aminocyclopropane-1-carboxylic acid) (Ojuederie et al., 2019). This study also revealed that each plant species responded to the IAA-producing bacteria *Paenibacillus* sp. $BSR_{1-1}$ in different ways. The levels of endogenous IAA response to some stress conditions within each plant tissue may be different and receiving IAA from bacteria benefits plants when the endogenous IAA is below the optimum level for plant growth (Glick, 2012). Thus, the *Paenibacillus* sp. $BSR_{1-1}$ used in this study stimulated the growth of economic crops to different extents because each plant has different endogenous IAA levels. Moreover, the endogenous IAA level could be altered under water stress. Consequently, these crops responded to bacterial inoculation in different ways. For example, peanut growth decreased in the presence of salt stress. Inoculation of peanuts with the *Rhizobium japonicum* strain USDA-110 could alter the level of IAA in peanuts resulting in normal growth (Asim et al., 2013).

Drought usually inhibits photosynthesis in plants due to the photosynthesis pigment being destroyed by reactive oxygen species (Vurukonda et al., 2016). The lower level of chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents in leaves have been reported in several cultivated plants under drought stress, including sunflower

Table 4
*The number of root nodules and pods of peanut under full and low water conditions with and without* Paenibacillus *sp.* $BSR_{1-1}$

| | Number of root nodules | Number of pods |
|---|---|---|
| Low water + WH | 12.3±7.6 | 1.5±0.4 |
| Full water + WH | none | none |
| Low water + $BSR_{1-1}$ | 5.6±1.6 | 1.8±0.2 |
| Full water + $BSR_{1-1}$ | 2.3±0.9 | 1.0±0.0 |

*Note*. Abbreviations: WH = Water hyacinth; $BSR_{1-1}$ = *Paenibacillus* sp. $BSR_{1-1}$

(Manivannan et al., 2007) and chickpea before flowering (Mafakheri et al., 2010). In this study, the chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents in the rice leaves grown under low water conditions were decreased in the presence or absence of *Paenibacillus* sp. $BSR_{1-1}$. Additionally, the low water condition did not affect the chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents in peanut and sweet corn leaves. However, the chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents in the leaves of peanut and sweet corn grew under low water conditions were higher than those grown under full water conditions (Table 3). The reason for this is not known.

The soil used in this study was acidic soil that had low organic matter (Table 5). Therefore, cropping the soil with peanut, rice, and sweet corn could increase soil fertility when considering the available phosphorus, exchangeable potassium, and exchangeable magnesium. The amounts of available phosphorus, exchangeable potassium, and exchangeable magnesium in the soil planted with peanut, rice, and sweet corn were higher than in the unplanted soil. However, the amount of soil organic matter and the available phosphorus, exchangeable potassium, exchangeable calcium, and exchangeable magnesium in the soil inoculated with *Paenibacillus* sp. $BSR_{1-1}$ did not differ from the planted soil without bacterial inoculation.

## CONCLUSION

*Paenibacillus* sp. $BSR_{1-1}$ immobilized with water hyacinth has the potential to stimulate the growth of economic crops under low water conditions. In addition, early flowering and fruiting were seen for peanut and rice. However, further studies with low water conditions should be conducted for agricultural purposes.

Table 5
*Characteristics of soil in low water condition at the end of the experiment*

| | pH | Organic matter (g/kg) | % sand | % silt | % clay | Available phosphorus (mg/kg) | Exchangeable potassium (mg/kg) | Exchangeable calcium (mg/kg) | Exchangeable magnesium (mg/kg) |
|---|---|---|---|---|---|---|---|---|---|
| Soil at beginning of the experiment | 4.62 | 6.13 | 48 | 26 | 26 | 11.0 | 70 | 929 | 100 |
| Soil planted with peanut | 4.72 | 6.48 | 58 | 22 | 20 | 21.4 | 82 | 806 | 114 |
| Soil planted with peanut + *Paenibacillus* sp. $BSR_{1-1}$ | 4.72 | 6.78 | 56 | 24 | 20 | 20.4 | 80 | 831 | 105 |
| Soil planted with sweet corn | 4.58 | 6.45 | 58 | 21 | 21 | 32.0 | 167 | 822 | 119 |
| Soil planted with sweet corn + *Paenibacillus* sp. $BSR_{1-1}$ | 4.54 | 6.15 | 56 | 22 | 22 | 21.0 | 182 | 855 | 123 |
| Soil planted with rice | 4.69 | 7.47 | 54 | 22 | 24 | 27.5 | 163 | 956 | 134 |
| Soil planted with rice + *Paenibacillus* sp. $BSR_{1-1}$ | 4.64 | 7.12 | 50 | 25 | 25 | 16.3 | 179 | 967 | 131 |

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## REFERENCES

Asim, M., Aslam, M., Bano, A., Munir, M., Majeed, A., & Abbas, S. H. (2013). Role of phytohormones in root nodulation and yield of peanut under salt stress. *American Journal of Research Communication*, *1*(5), 191-208.

Bashan, Y. (1998). Inoculant of plant growth promoting bacteria for use in agriculture. *Biotechnology Advances*, *16*(4), 729-770. https://doi.org/10.1016/S0734-9750(98)00003-2

Brito, S. L., Santos, A. B., Barbosa, D. D., Fernandes, P. D., Fernandes-Júnior, P. I., & Lima L. M. (2019). *Bradyrhizobium* spp. as attenuators of water deficit stress in runner peanut genotypes based on physiological and gene expression responses. *Genetics and Molecular Research*, *18*(4), gmr18379. https://doi.org/10.4238/gmr18379

de Souza, R., Ambrosini, A., & Passaglia, L. M. P. (2015). Plant growth-promoting bacteria as inoculants in agricultural soils. *Genetics and Molecular Biology*, *38*(4), 401-419. https://doi.org/10.1590%2FS1415-475738420150053

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., & Basra, S. M. A. (2009). Plant drought stress: Effects, mechanisms and management. *Agronomy for Sustainable Development*, *29*, 185-212. https://doi.org/10.1051/agro:2008021

Food and Agriculture Organization of the United Nations. (n.d.). *Drought*. FAO. http://www.fao.org/emergencies/emergency-types/drought/en/

George, S. P., Ahmad, A., & Rao, M. B. (2001). Studies on carboxy methyl cellulose produced by an alkalothermophilic actinomycete. *Bioresource Technology*, *77*(2), 171-175. https://doi.org/10.1016/S0960-8524(00)00150-4

Glick, B. R. (2012). Plant growth-promoting bacteria: Mechanisms and applications. *Scientifica*, *2012*, 963401. https://doi.org/10.6064/2012/963401

Gusain, Y. S., Singh, U. S., & Sharma, A. K. (2014). Enzymatic amelioration of drought stress in rice through the application of plant growth promoting rhizobacteria (PGPR). *International Journal of Current Research*, *6*(1), 4487-4491.

Huang, X., El-Alawi, Y., Penrose, D. M., Glick, B. R., & Greenberg, B. M. (2004). Response of three grass species to creosote during phytoremediation. *Environmental Pollution*, *130*(3), 453-363. https://doi.org/10.1016/j.envpol.2003.12.018

Joshi, B., Chaudhary, A., Singh, H., & Kumar, P. A. (2020). Prospective evaluation of individual and consortia plant growth promoting rhizobacteria for drought stress amelioration in rice (*Oryza sativa* L.). *Plant Soil*, *457*, 225-240. https://doi.org/10.1007/s11104-020-04730-x

Khan, N., & Bano, A. (2019). Exopolysaccharide producing rhizobacteria and their impact on growth and drought tolerance of wheat grown under rainfed conditions. *PLOS One*, *14*(9), e0222302. https://doi.org/10.1371/journal.pone.0222302

Kirdponpattara, S., Chuetor, S., Sriariyanun, M., & Phisalaphong, M. (2021). Bioethanol production by *Pichia stipites* immobilized on water hyacinth and thin shell silk cocoon. *Applied Science and Engineering Progress*, *15*(3). https://doi.org/10.14416/j.asep.2021.03.006

Kumar, B. L., & Gopal, D. V. R. S. (2015). Effective role of indigenous microorganisms for sustainable environment. *3 Biotech*, *5*(6), 867-876. https://doi.org/10.1007/s13205-015-0293-6

Lu, J., Peng, W., Lv, Y., Jiang, Y., Xu, B., Zhang, W., Zhou, J., Dong, W., Xin, F., & Jiang, M.

(2020). Application of cell immobilization technology in microbial cocultivation systems for biochemicals production. *Industrial and Engineering Chemistry Research*, *59*(39), 17026−17034. https://doi.org/10.1021/acs.iecr.0c01867

Mafakheri, A., Siosemardeh, A., Bahramnejad, B., Struik, P. C., & Sohrabi, Y. (2010). Effect of drought stress on yield, proline and chlorophyll contents in three chickpea cultivars. *Australian Journal of Crop Science*, *4*(8), 580-585.

Manivannan, P., Abdul Jaleel, C., Sankar, B., Kishorekumar, A., Somasundaram, R., Lakshmanan, G. M. A., & Panneerselvam R. (2007). Growth, biochemical modifications and proline metabolism in *Helianthus annuus* L. as induced by drought stress. *Colloids and Surfaces B: Biointerfaces*, *59*(2), 141-149. https://doi.org/10.1016/j.colsurfb.2007.05.002

Martins, S. C. S., Martins, C. M., Fiúza, L. M. C. G., & Santaella, S. T. (2013). Immobilization of microbial cells: A promising tool for treatment of toxic pollutants in industrial wastewater. *African Journal of Biotechnology*, *12*(28), 4412-4418. https://doi.org/10.5897/AJB12.2677

Mohr, H., & Schopfer, P. (1995). *Plant physiology*. Springer-Verlag.

Naseem, H., & Bano, A. (2014). Role of plant growth-promoting rhizobacteria and their exopolysaccharide in drought tolerance of maize. *Journal of Plant Interactions*, *9*(1), 689-701. https://doi.org/10.1080/17429145.2014.902125

Niu, X., Song, L., Xiao, Y., & Ge, W. (2018). Drought-tolerant plant growth promoting rhizobacteria associated with foxtail millet in a semi-arid agroecosystem and their potential in alleviating drought stress. *Frontiers in Microbiology*, *8*, 2580. https://doi.org/10.3389/fmicb.2017.02580

Ojuederie, O. B., Olanrewaju, O. S., & Babalola, O. O. (2019). Plant growth promoting rhizobacterial mitigation of drought stress in crop plants: Implications for sustainable agriculture. *Agronomy*, *9*(11), 712. https://doi.org/10.3390/agronomy9110712

Partovinia, A., & Rasekh, B. (2018). Review of the immobilized microbial cell systems for bioremediation of petroleum hydrocarbons polluted environments. *Critical Reviews in Environmental Science and Technology*, *48*(1), 1-38. https://doi.org/10.1080/10643389.2018.1439652

Penrose, D. M., & Glick, B. R. (2003). Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiologia Plantarum*, *118*(1), 10-15. https://doi.org/10.1034/j.1399-3054.2003.00086.x

Pérez-Miranda, S., Cabirol, N., George-Téllez, R., Zamudio-Rivera, L. S., & Fernández, F. J. (2007). O-CAS, a fast and universal method for siderophore detection. *Journal of Microbiological Methods*, *70*(1), 127-131. https://doi.org/10.1016/j.mimet.2007.03.023

Prajapati, K. B., & Modi, H. A. (2012). Isolation and characterization of potassium solubilizing bacteria from ceramic industry soil. *CIBTech Journal of Microbiology*, *1*(2-3), 8-14. https://doi.org/10.13140/RG.2.2.14843.95525

Sahin, U., Ekinci, M., Kiziloglu, F. M., Yildirim, E., Turan, M., Kotan, R., & Ors, S. (2015). Ameliorative effects of plant growth promoting bacteria on water-yield relationships, growth, and nutrient uptake of lettuce plants under different irrigation levels. *HortScience*, *50*(9), 1379-1386. https://doi.org/10.21273/HORTSCI.50.9.1379

Santos, M. S., Nogueira, M. A., & Hungria, M. (2019). Microbial inoculants: Reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. *AMB Express*, *9*, 205. https://doi.org/10.1186/s13568-019-0932-0

Shultana, R., Kee Zuan, A. T., Yusop, M. R., & Saud, H. M. (2020b). Characterization of salt-tolerant plant growth-promoting rhizobacteria and the effect on growth and yield of saline-affected rice. *PLOS One*, *15*(9), e0238537. https://doi.org/10.1371/journal.pone.0238537

Shultana, R., Kee Zuan, A. T., Yusop, M. R., Saud, H. M., & Ayanda, A. F. (2020a). Effect of salt-tolerant bacterial inoculations on rice seedlings differing in salt-tolerance under saline soil conditions. *Agronomy*, *10*(7), 1030. https://doi.org/10.3390/agronomy10071030

Somtrakoon, K., Sangdee, A., & Chouychai, W. (2019). Roles of plant growth promoting bacteria on growth of ornamental plants grown in anthracene-spiked soil. *Journal of Agricultural Research and Extension*, *36*(2), 11-22.

Somtrakoon, K., Sangdee, A., & Chouychai, W. (2022). Maintaining growth of aquatic morning glory under drought condition by *Paenibacillus* sp. $BSR_{1-1}$. *Trends in Sciences*, *19*(5), 2884. https://doi.org/10.48048/tis.2022.2884

Sun, L., Yang, Y., Wang, R., Li, S., Qiu, Y., Lei, P. Gao, J., Xu, H., Zhang, F., & Lv, Y. (2020). Effects of exopolysaccharide derived from *Pantoea alhagi* NX-11 on drought resistance of rice and its efficient fermentation preparation. *International Journal of Biological Macromolecules*, *162*, 946-955. https://doi.org/10.1016/j.ijbiomac.2020.06.199

Truong, T. H. H., & Marschner, P. (2018). Respiration, available N and microbial biomass N in soil amended with mixes of organic materials differing in C/N ratio and decomposition stage. *Geoderma*, *319*, 167-174. https://doi.org/10.1016/j.geoderma.2018.01.012

Vurukonda, S. S. K. P., Vardharajula, S., Shrivastava, M., & SkZ, A. (2016). Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria. *Microbiological Research*, *184*, 13-24. https://doi.org/10.1016/j.micres.2015.12.003

Wu, S., Shen, Z., Yang, C., Zhou, Y., Li, X., Zeng, G., Ai, S., & He, H. (2017). Effects of C/N ratio and bulking agent on speciation of Zn and Cu and enzymatic activity during pig manure composting. *International Biodeterioration and Biodegradation*, *119*, 429-436. https://doi.org/10.1016/j.ibiod.2016.09.016

Yao, W., Wu, X., Zhu, J., Sun, B., Zhang, Y. Y., & Miller, C. (2011). Bacterial cellulose membrane - A new support carrier for yeast immobilization for ethanol fermentation. *Process Biochemistry*, *46*(10), 2054-2058. https://doi.org/10.1016/j.procbio.2011.07.006

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# Effect of *Azolla filiculoides* Meal Inclusion in the Napier Silage Total Mixed Ration on the *In vitro* Cumulative Gas Production and Digestibility

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## ABSTRACT

This study was carried out to determine the nutritional value and digestibility of total mixed ration (TMR) Napier silage with different *Azolla filiculoides* meal inclusion percentages. Samples of *Azolla* were cultivated in the tank with the media from 1.0 g/L dilution of sheep manure. Inclusion of 0% (control), 6% (T1), 10% (T2), 16% (T3), and 23% (T4) *A. filiculoides* meal was used to replace the proportion of Napier silage and soybean meal according to treatments with four replicates. All treatments were analyzed to determine the nutritional composition, and *in vitro* gas production was recorded for 96 h. In contrast, *in vitro* dry matter digestibility (IVDMD), *in vitro* organic matter digestibility (IVOMD), and metabolizable energy (ME) of each TMR mixture were determined using the published equation. As a result, only T4 had shown a significant difference ($p$<0.05) in crude protein (CP) and ether extract (EE) compared to other treatments. Values of dry matter (DM), CP, and ash of the TMRs were not affected on T1, T2, T3, and control. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were increased significantly at T3 and T4 compared to other treatments even though higher ($p$<0.05) acid detergent lignin (ADL) as replacement of 5.0% Napier silage and 1.0% soybean meal had shown a competitive value in their nutritional compared to the common TMR for ruminants. Therefore, a fermentation process was suggested to degrade indigestible components of *A. filiculoides* to enhance the potential of this species as an alternative feed source for a ruminant.

*Keywords*: *Azolla filiculoides*, digestibility, *in vitro* gas production, ruminant, total mixed rations

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## INTRODUCTION

Agriculture has become one of the fundamental industries in Malaysia. These industries had contributed lucrative employment and concurrently supplied the domestic food requirements for the population. This local industry establishment will ensure food security for domestic consumption and reduces dependency on imported livestock product. The livestock industry has contributed around 12.4% of total agricultural gross domestic product (GDP) in 2013, whereby the ruminant sub-sector had only contributed 12.1% from it (Shanmuganvelu, 2014). Malaysian National Agro-food Policy 2011–2020 (NAP) had emphasized the demand and production of meat which is expected to be increased. From 2010 to 2020, an increment of local demand for meat is estimated from 1.4 million metric tons (MT) to 1.8 million MT with a growth of 2.4% per annum, while meat production is forecast to increase from 1.6 million MT to 2.1 million MT with a growth of 2.7% per annum in the same period. The demand increase is also expected for other livestock products such as milk and eggs. However, the ruminant sector, which consists of beef and dairy cattle, dairy, buffaloes, sheep, and goats, is still small-scale (Rosali, 2015). Positive progress has been observed in recent years, but it can still not meet the local demand. Thus, Malaysia imports most of the needed beef, mutton, and dairy product from abroad, especially India, Australia, and New Zealand, to cater to the shortage. In 2014, the level of self-sufficiency (SSL) for beef, mutton, and milk were 24.84%, 13.10%, and 12.93%, respectively. The lag in this ruminant sector is normally associated with several factors such as the lack of land resources, high feed price, cheaper import substitutes, poor private sector involvement, disease prevention and control, and lack of quality breeds, expertise, and workforce (National Agro-food Policy 2011–2020). The insufficient local protein source for the domestic market and high dependency on imported meats are associated with the issues regarding Malaysia's ruminant industry, especially in feeds and production systems. Eventually, research and development of any abundance material or local by-product had been emphasized to ensure our ruminant industry could be viable and sustainable for our domestic consumption.

Components of nutrient requirement were based on animal species, and stages had been highlighted in the research in developing new feed for livestock. The fiber source that farmers had used was from local agriculture by-products such as oil palm frond (OPF), corn stalk, and bagasse, while fish meal, copra cake, and soybean meal were used as a protein source in the feed. Palm kernel cake (PKC) or palm oil sludge (POS) was also used as an alternative source of protein and energy for the animal (Kum & Zahari, 2011; Seephueak et al., 2011). POS is a by-product from the palm oil mill effluent (POME) filtration that consists of approximately 9.6%–16.0% CP (Devendra et al., 1983). However, due to some changes in the livestock production systems towards semi-intensive and fully intensive systems,

agriculture and industrial waste were highly demanded and became pricey in the market. Indeed, the availability of these products was on a seasonal basis, and the supply was unable to be sustained due to higher prices was offered by the exporter to support a huge industry such as construction, papers, and cosmetics (Akbari & Resalati, 2012, Kumar et al., 2020; Sahota, 2014). Eventually, farmers had chosen Napier grass and soybean meal as the main source of fiber and protein, respectively. Although Napier grass has become one of the renewable fiber sources, it requires areas and workforce to ensure an adequate amount of quality fodder could be produced. In addition, shortages of labor and the inability to manage the cutting interval at 6 to 8 weeks had decreased the fodder nutritional quality (Zailan et al., 2016b). Meanwhile, due to the runaway prices, farmers and feed producers had to reduce or replace the soybean meal in their feed formulation with other alternative ingredients, such as palm kernel cake, even though its availability in the market is relatively limited and its price is unstable. Therefore, an effort was made to discover an alternative source of fiber and protein that is practical and affordable for farmers to produce.

Meanwhile, most animal farms will have a drainage system that drains farm waste to the main canal. All drainage was predominant by several aquatic plant species such as *Eichhornia crassipes*, *Pistia stratiotes*, and *Azolla filiculoides*. Those species had been found necessary as bioremediation agents and bio-fertilizers, which have an important role in ecology conservation (Escoto et al., 2019). However, the uncontrolled population of the floating aquatic plants has been reported to harm aquatic ecology. Therefore, previous researchers have realized the potential of these plants as a source of additional fiber and protein for livestock. Hence, studies related utilization of an aquatic plant as an animal feed were conducted many years before. However, *A. filiculoides* species was found to be more suitable than other aquatic plant species due to its growth potential and nutrient content (Kamaruddin et al., 2019). In an optimal environment, this species can achieve a doubling time of 2–7 days and produce up to 2.9 $g/m^2$ $day^{-1}$ with a crude protein (CP) content of 22.48% $kg^{-1}$ DM, crude fiber (CF), 14.70% $kg^{-1}$ DM, neutral detergent fiber (NDF) 37.6% $kg^{-1}$ DM, and acid detergent lignin (ADL) 8.03% $kg^{-1}$ DM (Kollah et al., 2016). This species was also able to be cultivated in the livestock manure liquid. The bio-phytoremediation role was proved to absorb up to 2.6 tons N/ha $year^{-1}$ and 0.43 tons P/ha $year^{-1}$ from the 'farm waste treatment collector pond' before being drained into the main drainage system (Costa et al., 1999). In this environment, 1.5 $g/m^2$ $day^{-1}$ can be harvested every 14 days with nutrient composition of CP 21.3 $\%kg^{-1}$ DM, CF 16.4 $\%kg^{-1}$ DM, NDF 37.6 $\%kg^{-1}$ DM, ADF 27.64 $\%kg^{-1}$ DM and ADL 8.03 $\%kg^{-1}$ DM (Mohammad Fitri Rimi et al., 2021). Therefore, farmers will be able to maximize the use of existing resources in the farm to reduce the production cost. The objective of this study was to investigate the effect of

different levels of *A. filiculoides* meal as a fiber and protein source in a ruminant diet through *in vitro* gas production and feed degradability trials.

## METHODS AND MATERIALS

### Research Area

The study was conducted at Livestock Science Research Center MARDI headquarters, Serdang, Selangor (2°59' 23"N 101°42'08"E) and MARDI's Livestock Centre of Excellence, Kluang, Johor (1°56'58"N 103°21'54"E). The cultivation location of *Azolla filiculoides* was conducted at the MARDI Serdang pasture study plot (2°59'23"N 101°41'43"E). At the same time, the mixing activity of total mixed ration and laboratory analysis was carried out at the MARDI feed bio-process incubator, Serdang (2°59'01''N 101°42'06''E). Meanwhile, rumen fluid was collected from cannulated animal husbandry of Kluang MARDI Research Station (1°57'27"N 103°21'35"E), and digestion studies were conducted at digestibility laboratory at Kluang MARDI Research Station (1°56'56"N 103°21'56''E).

### Cultivation and Preparation of *Azolla filiculoides* Meal

Cultivation of *A. filiculoides* was conducted in the five units' canvas pools with 2.5m x 2.5m. All pools are placed in the opened area and directly exposed to sunlight. After filling the water approximately 1.0 m deep, all pools were left for 24 h. Sheep manure was measured into 6.3 kg for each pool and was soaked until it spread evenly. Subsequently, approximately 100 grams of fresh *A. filiculoides* were spread into each pool. After 14 days of cultivation, harvesting was carried out using sieve containers to remove the water from the plant. Next, all harvested plants were dried using a forced air oven at 60 °C for 72 h and ground into a 1 mm size *Azolla* meal. Finally, the grounded sample was packed into a dry and clean container for storage in the 2 °C chillers.

Samples had been analyzed to determine the composition of total dry matter (DM), organic matter (OM), crude protein (CP), crude fiber (CF), ether extract (EE), and ash (IM) as guided in the Association of Official Analytical Chemists (AOAC) (2005) and the component of fiber (NDF, ADF, and ADL) was measured using fiber cap and fiber tech distillation machine. According to Van Soest et al. (1991), those measurement principles were made. The same procedures were used to determine the nutrient composition and fiber components of other materials used in this treatment.

### Preparation of Napier Silage and Total Mixed Ration

The harvested Napier grass was wilted for 24 h and chopped into 2–4 cm before being ensilaged in the plastic drum for 21 days. All drums were prepared for the adaptation process of the cannulated animal, and it had been stored under the shaded area. The formulation was calculated based on the nutritional value of each material by setting the homogeneity on CP and ether

extract (EE) composition. The rations met the nutritional requirements for maintenance cattle (National Research Council [NRC], 2001). Then, all ingredients were mixed for 10 minutes using a 100 kg industrial horizontal mixer machine. Five total mixed rations (TMR) were formulated based on the percentage of the inclusion of *Azolla filiculoides* meal which is 0% (control), 6% (T1), 10% (T2), 17% (T3), and 23% (T4). Sample from each TMR was taken for the proximate and fiber analysis. The actual nutritional composition for each treatment is shown in Table 1.

Table 1

*Ingredients of the total mixed rations (TMR) with the different inclusion percentages of Azolla filiculoides meal*

| | TMR | | | | |
|---|---|---|---|---|---|
| Indices | Control | T1 | T2 | T3 | T4 |
| Ingredients | | | | | |
| Molasses (%) | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| Palm oil (%) | 3.3 | 3.3 | 3.3 | 3.3 | 3.3 |
| Salt (%) | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Mineral and vitamin (%) | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| Limestone (%) | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Maize (%) | 13.5 | 13.5 | 13.5 | 13.5 | 13.5 |
| Napier silage (%) | 60.0 | 55.0 | 52.0 | 47.0 | 41.0 |
| Soybean meal (%) | 17.5 | 16.5 | 15.5 | 14.5 | 13.5 |
| *Azolla filiculoides* meal (%) | 0.0 | 6.0 | 10.0 | 16.0 | 23.0 |

*Note*. Control = 0% *Azolla* meal; T1 = 6% *Azolla* meal; T2 = 10% *Azolla* meal; T3 = 16% *Azolla* meal; T4 = 23% *Azolla* meal

**Chemical Analysis**

Samples from each TMR were analyzed for DM, CP, EE, CF, and ash according to the Association of Official Agricultural Chemists (AOAC) (1975). In addition, the leaf samples were analyzed for NDF, ADF, ADL, and cellulose according to Van Soest et al. (1991).

**Donor Animal's Inocula**

The adaptation process on three (3) cannulated bulls was carried out for 14 days. The cannulated bulls were fed with Napier silage and a total mixed ration containing 23% *Azolla* meal throughout the adaptation period. Napier silage and total mixed ration were given two times a day at a ratio of 55:45 according to the rate of 3% of the individual bodyweight and material DM.

Instruments to collect the rumen liquid were prepared a day before because it is recommended to make a collection in the morning before feeding the animals. Thermos (filled with hot water), polyvinyl chloride (PVC) perforated strainer pole, and carbon dioxide ($CO_2$) tank had been prepared a day before the water tub had been set up at 39 °C and the buffer solution was ready for the rumen liquid. Meanwhile, the bull was restrained while the rumen liquid collecting equipment was inserted into the rumen. Rumen liquid from the cannulated bulls was pooled in the flask. At the same time, it has been flushed using the $CO_2$ to maintain an anaerobic environment for the rumen microorganism until it is poured into the prepared buffer solution.

**Gas Production Assay**

According to Theodorou et al. (1994), the gas production assay was carried out. Approximately 30 mL buffer media was filled with 200 mg samples in the syringe. An arrangement of the syringe was according to randomized complete block design (RCBD) in the water tub. Anaerobic buffer solution, which is contained micro and macro elements reducing agent and a reduction indicator of resazurin, was added to the bottles containing 10 mL of ruminal fluid. Negative controls (blank) containing buffered rumen fluid but no substrates were also included in triplicate to correct gas produced from small particles present in the ruminal fluid. Cumulative gas production (mL/g DM) was recorded at 2, 4, 6, 8, 10, 12, 15, 19, 24, 30, 36, 48, 72, and 96 h after incubation at 39 °C. The volume of gas produced after 24 h of incubation (GP 24) was used as an index of the energy feed value of tree fodder samples (Menke, 1988). The volume of gas produced (GP) (mL 200 $mg^{-1}$ DM) after 24 h of incubation was used with CP content to estimate metabolizable energy (ME) concentration (MJ $kg^{-1}$ DM) based on the following equation reported by Menke et al. (1979) for roughage feeds:

$$ME = 2.2 + 0{:}1357\ GP + 0{:}057\ CP + 0{:}002859\ CP^2 \quad (R^2 = 94\%;\ n = 200)$$

where,

ME = Metabolisable energy (MJ $kg^{-1}$ DM)

GP = Gas production after 24 h (mL 200 $mg^{-1}$ DM)

CP = Crude protein (%)

***In vitro* Degradability**

At the end of incubation (96 h), the contents of each syringe were completely discarded from the syringe in the 100 mL centrifuge tube. Fermentation residues were dried at 105 ºC overnight and then incinerated in a muffle furnace at 550 ºC for 12 h. Loss in weight after incineration was used as a measure of ash. The *in vitro* organic matter degradability (IVOMD) at 96 h of incubation was calculated as equation below:

$$IVDMD\ (\%) = [(DM\ sample - DM\ residue - blank) / DM\ sample] \times 100$$

where,
IVDMD = *In vitro* dry matter digestibility
DM = Dry matter

The tubes were centrifuged at 20,000 x *g* for 15 min, and 15 mL of supernatant was kept for VFA determination following the procedure described by Cottyn & Boucque (1968). First, the pellets were dried in a forced-air oven at 60 °C for 48 h to determine the residual DM weights. Then, to determine ash content, the residues were kept at 550 °C for 8 hours to estimate organic matter (OM). Finally, *in vitro,* organic matter digestibility was calculated as the OM, which disappeared from the initial weight inserted into the tube. Calculations were as follows:

IVOMD (%) = [(OM sample – OM residue – blank) / OM sample] x 100

where,
IVOMD = *In vitro* organic matter digestibility
OM = Organic matter

Next, the supernatant was separated from the residue. Then, the mixture obtained from *in vitro* analysis was put into a centrifuge tube and then centrifuged at 2,500 x *g* in the 4 °C for 30 min. Finally, the supernatants were transferred into a vial in 4 replicates each treatment and stored in the -20 °C freezer for the next procedure.

**Analysis of volatile fatty acid (VFA) using Gas Chromatography (GC).** The samples were thawed for 1 h before arranging the vial in the GC. Analyses were conducted on a 6820-gas chromatograph system from Agilent Technologies (USA). The instruments were prepared with a free fatty acid phase (FFAP) capillary column, 30 m x 250 µm x 0.25 µm (Quadrex Corporation, USA) and using carrier gas that could flow nitrogen gas at 1.0 mL/minute with the flame ionization detector (FID). The temperature was programmed using 60–200 °C (20 °C/min, 10 min) with the injector—250 °C and detector—300 °C. The injector was equipped with a glass liner of glass wool to separate dirt particles from the sample. The samples were dosed by an HT 300A automatic dosing device (Agilent Technology, USA) at an injection size of 1 µl using the split method and a 30:1 splitting ratio and the analysis time is approximately 15 min.

**Statistical Analysis**

To assess the replacement and inclusion effect of Napier silage and soybean meal with *A. filiculoides* meal on the nutrient composition, GP, IVDMD, IVOMD, and ME of Napier silage TMR, a 5 x 4 factorial analysis of variance (ANOVA) was conducted. The means and standard error of means (SEM) for five different inclusions of *A. filiculoides* in the Napier silage TMR as a function of the two factors are presented in Tables 2 and 3. In addition, the *F* test and Duncan's test for post-hoc comparisons ($p<0.05$) were applied. All

statistical analyses were performed using the SPSS (version 25) software package.

## RESULTS

An actual nutrients composition value of TMRs has shown in Table 2. Soybean meal and Napier silage had become the main source of protein and fiber in this TMR. The inclusion of *A. filiculoides* meal into the TMR did not affect ($p$>0.05) their DM, CP, and ash compared to the control. However, the inclusion of 23.0% of *A. filiculoides* meal (T4) replacing 4.0% of soybean meal and 19.0% of Napier silage from the TMR had significantly affected the values of CF, OM, and EE compared to the treatment that consisted of 0% *A. filiculoides* meal inclusion. The values of CF and OM was significantly higher ($p$<0.05) while EE had reduced significantly compared to the control. Besides, the values of NDF, ADF, and ADL showed an increment ($p$<0.05) at the range of 9.3%–21.0%, with the 10% inclusion of *A. filiculoides* meal (T2) replacing 3 % of soybean meal and 13% of Napier silage.

The effect of replacing Napier silage and soybean meal with *A. filiculoides* meal into the ruminant diet on the cumulative *in vitro* gases production, IVDMD, IVOMD, and ME was as shown in Table 3 and Figure 1. At 6% of *A. filiculoides* (T1), cumulative i*n vitro* gas production at 24, 48, and 96 h was significantly higher than T2, T3, and T4 during the incubation period. However, after 48 h incubation, the gas production was still significantly increased at the higher inclusion treatments (T2, T3, and T4) instead of T1, which had nearly reached a plateau after that period. From the result, the highest volume of gasses was produced at 6% inclusion (T1), and the lowest was obtained from 23% inclusion (T4) which is 261.2 mL $g^{-1}$ DM and 228.3 mL $g^{-1}$ DM, respectively.

Table 2

*Nutrient composition and fiber components (%/DM basis) of the total mixed rations (TMR) with the different inclusion percentage of* Azolla filiculoides *meal*

| Indices | TMR | | | | |
|---|---|---|---|---|---|
| | Control (n = 4) | T1 (n = 4) | T2 (n = 4) | T3 (n = 4) | T4 (n = 4) |
| Nutrient composition | | | | | |
| Dry matter (%) | 58.5 ± 2.7$^{a}$ | 57.8 ± 1.8$^{a}$ | 58.8 ± 2.8$^{a}$ | 64.0 ± 1.4$^{a}$ | 66.5 ± 2.1$^{a}$ |
| Organic matter (%) | 31.1 ± 3.3$^{c}$ | 34.8 ± 0.8$^{bc}$ | 35.7 ± 2.9$^{abc}$ | 38.9 ± 1.5$^{ab}$ | 42.2 ± 2.1$^{a}$ |
| Crude protein (%) | 15.2 ± 0.3$^{a}$ | 14.5 ± 0.6$^{a}$ | 14.9 ± 1.3$^{a}$ | 15.2 ± 0.4$^{a}$ | 15.4 ± 0.2$^{a}$ |
| Crude fiber (%) | 27.2 ± 0.3$^{b}$ | 26.8 ± 0.1$^{b}$ | 28.4 ± 0.6$^{ab}$ | 29.0 ± 0.3$^{ab}$ | 30.3 ± 0.5$^{a}$ |
| Ether extract (%) | 6.3 ± 0.2$^{a}$ | 6.2 ± 0.1$^{a}$ | 6.1 ± 0.1$^{a}$ | 5.2 ± 0.6$^{bc}$ | 4.2 ± 0.6$^{c}$ |
| Ash (%) | 27.5 ± 0.7$^{a}$ | 23.0 ± 1.8$^{a}$ | 23.1 ± 1.5$^{a}$ | 25.1 ± 1.6$^{a}$ | 24.1 ± 1.0$^{a}$ |

Table 2 *(Continue)*

| Indices | TMR | | | | |
|---|---|---|---|---|---|
| | Control (n = 4) | T1 (n = 4) | T2 (n = 4) | T3 (n = 4) | T4 (n = 4) |
| Fiber components | | | | | |
| NDF (%) | 31.0 ± 0.6$^{c}$ | 32.5 ± 0.5$^{bc}$ | 33.7 ± 0.6$^{b}$ | 36.3 ± 0.5$^{a}$ | 37.5 ± 0.1$^{a}$ |
| ADF (%) | 24.6 ± 0.4$^{b}$ | 25.0 ± 0.2$^{b}$ | 25.0 ± 0.2$^{b}$ | 26.6 ± 0.3$^{a}$ | 26.9 ± 0.5$^{a}$ |
| ADL (%) | 12.6 ± 0.5$^{b}$ | 13.0 ± 0.4$^{ab}$ | 14.0 ± 0.3$^{ab}$ | 14.1 ± 0.2$^{ab}$ | 14.2 ± 0.3$^{a}$ |

*Note*. Control = 0% *Azolla filiculoides* meal; T1 = 6% *Azolla filiculoides* meal; T2 = 10% *Azolla filiculoides* meal; T3 = 16% *Azolla filiculoides* meal; T4 = 23% *Azolla filiculoides* meal; n = Number of samples; NDF = Neutral detergent fiber; ADF = Acid detergent fiber; ADL = Acid detergent lignin
All data are means ± standard error of the mean (S.E.M.)
$^{a,b,c}$ Mean with different superscripts within a row are significantly different ($p$<0.05)

IVDMD was significantly affected by the increase of *A. filiculoides* inclusion percentage in the TMR formulation. From the result, a dry matter digestibility was reduced at 12.2%–41.2% after replacing the Napier silage and soybean meal with *A. filiculoides* meal. With the inclusion, the highest dry matter digestibility was determined at 391.1 g kg$^{-1}$ DM at 6% inclusion (T1), and the lowest was recorded at 262.0 g kg$^{-1}$ DM, which is from T4. However, IVOMD and ME were not affected with the lower inclusion (T1). The inclusion of 10% (T2) and above had resulted in a significant reduction on both parameters. The highest IVOMD of the TMR with *A. filiculoides* inclusion was 453.6 g kg$^{-1}$ DM (T1 = 6%), and the lowest was 417.6 g kg$^{-1}$ DM (T4 = 23%). However, the values of ME were also directly reflected by the IVOMD. Inclusion of 6% *A. filiculoides* (T1) had produced higher energy for the metabolic process during digestion, similar with the control ($p$>0.05) compared to a higher percentage of inclusion. After *A. filiculoides* meal was used as a fiber and protein alternative source, the highest ME was recorded as 14.1 MJ kg$^{-1}$ DM (T1 = 6%), and the lowest was 11.6 MJ kg$^{-1}$ DM.

From this research, the concentration of total VFA and proportion of acetate, propionate, and butyrate were shown in Table 4. The basal diet (control) had produced 86.0 mM/L total VFA with the proportion of partial VFA (acetate, propionate, and butyrate) at 50.4:26.7:15.1, respectively. When viewed from all treatments, the inclusion of *A. filiculoides* meal in the ruminant feed had produced total VFA at 87.9 mM/L–120.0 mM/L. The difference in total VFA produced between treatments was significant. The value was increased as a higher percentage of *A. filiculoides* meal was used on the feed. As the inclusion percentage was increased, acetate and propionate of T3 and T4 were also enhanced significantly. However, propionate production was higher ($p$<0.05) at T1 and T2 than T3 and T4. From

this study, T4 had produced the highest concentration of acetate and butyrate, which are 67.4 mM/L and 18.1 mM/L, respectively. Propionate in T4 had significantly decreased ($p$<0.05) to 14.8 mM/L.

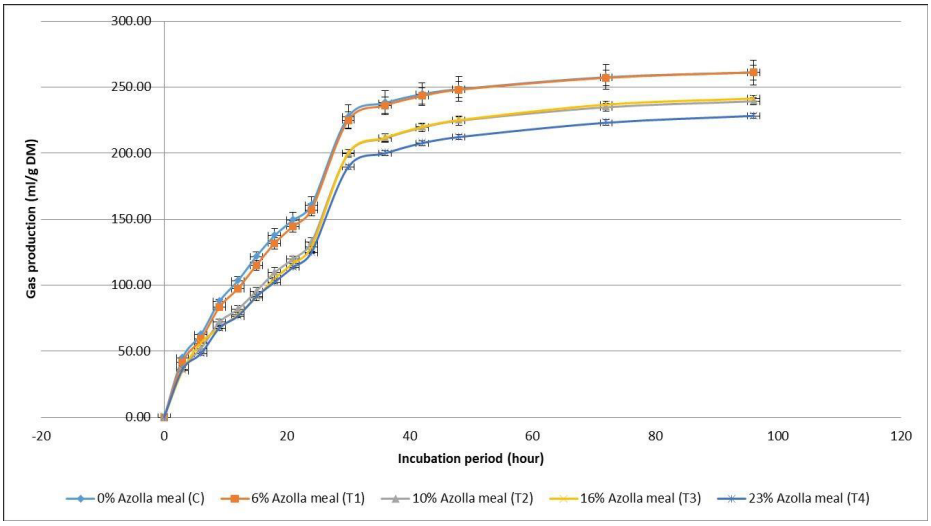


*Figure 1*. *In vitro* gas production profiles of total mixed rations with the different levels of *Azolla filiculoides* meal in the cattle rumen incubation

Concurrently, the acetate and propionate A:P ratio was determined, as shown in Figure 2. The ratio increased as more *A. filiculoides* meal inclusion was used to replace the portion of Napier silage and soybean meal. The A:P ratio produced from the trial was at 2.43 to 2.75, and the T1 had no significant difference with control. The treatment with more than 16% inclusion had significantly produced a higher A:P ratio than TMR without the inclusion of *A. filiculoides* meal inclusion.

Table 3

In vitro *fermentation characteristics of total mixed rations (TMR) with the different levels of* Azolla filiculoides *meal during* in vitro *ruminal incubation*

| Parameter | Inclusion of *Azolla* meal in the TMR | | | | |
|---|---|---|---|---|---|
| | Control (n = 4) | T1 (n = 4) | T2 (n = 4) | T3 (n = 4) | T4 (n = 4) |
| Gas | | | | | |
| GP 24 | 160.63 ± 6.21$^{a,z}$ | 157.03 ± 4.61$^{a,z}$ | 132.48 ± 3.24$^{b,z}$ | 128.73 ± 3.50$^{b,z}$ | 124.75 ± 0.86$^{b,z}$ |
| GP 48 | 248.50 ± 9.39$^{a,y}$ | 248.03 ± 6.25$^{a,y}$ | 224.58 ± 3.51$^{b,y}$ | 225.03 ± 2.50$^{b,y}$ | 212.18 ± 1.68$^{b,y}$ |
| GP 96 | 261.10 ± 9.29$^{a,y}$ | 261.23 ± 5.60$^{a,y}$ | 239.33 ± 2.86$^{b,x}$ | 241.35 ± 2.06$^{b,x}$ | 228.25 ± 1.84$^{b,x}$ |

Table 3 *(Continue)*

| Parameter | Inclusion of *Azolla* meal in the TMR | | | | |
|---|---|---|---|---|---|
| | Control (n = 4) | T1 (n = 4) | T2 (n = 4) | T3 (n = 4) | T4 (n = 4) |
| Degradability | | | | | |
| IVDMD (g/kg DM) | 445.5 ± 0.84[a] | 391.1 ± 0.75[b] | 339.0 ± 1.03[c] | 315.4 ± 0.88[c] | 262.0 ± 0.84[d] |
| IVOMD (g/kg DM) | 542.2 ± 0.23[a] | 453.6 ± 0.83[a] | 445.0 ± 0.01[b] | 421.0 ± 0.01[bc] | 417.6 ± 0.01[c] |
| ME (MJ/kg DM) | 14.40 ± 0.45[a] | 14.08 ± 0.26[a] | 12.50 ± 0.17[b] | 12.08 ± 0.29[b] | 11.60 ± 0.18[b] |

*Note*. GP = Gas production (mL/g DM at 24 hours ,48 hours, and 96 hours); IVDMD = *In vitro* dry matter degradability (g/kg DM); IVOMD = *In vitro* organic matter degradability (g/kg DM); ME = Metabolizable energy content (MJ/kg DM), n = Number of samples; IVDMD = *In vitro* dry matter digestibility; IVOMD = *In vitro* organic matter digestibility; ME = Metabolizable energy

All analyses are means ± standard error of the mean (S.E.M.)

[a,b,c] Mean with different superscripts within a row are significantly different ($p$<0.05)

[x,y,z] Mean with different superscripts within a column are significantly different ($p$<0.05)

Table 4

*Volatile fatty acid (VFA) profile of Napier silage total mixed ration with different inclusion percentage of* Azolla filiculoides *meal*

| VFA (mM/L) | Control (n = 4) | | T1 (n = 4) | | T2 (n = 4) | | T3 (n = 4) | | T4 (n = 4) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | ±SEM | Mean | ± SEM | Mean | ± SEM | Mean | ± SEM | Mean | ± SEM |
| Acetate | 50.4 | ± 1.47[b] | 52.8 | ± 2.04[b] | 55.3 | ± 2.22[b] | 64.2 | ± 1.49[a] | 67.4 | ± 2.81[a] |
| Propionate | 26.7 | ± 1.01[a] | 23.5 | ± 2.04[a] | 16.8 | ± 0.85[b] | 16.2 | ± 0.91[b] | 14.8 | ±0.36[b] |
| Butyrate | 15.1 | ± 0.23[b] | 15.3 | ± 0.27[b] | 15.6 | ± 0.26[b] | 17.3 | ± 0.37[a] | 18.1 | ± 0.60[a] |
| Total VFA | 86.0 | ±0.38[c] | 87.9 | ± 0.14[c] | 97.1 | ± 0.08[c] | 116.1 | ± 0.87[b] | 120.0 | ± 0.32[a] |

*Note*. VFA = Volatile fatty acid; Control = 0% *Azolla filiculoides* meal; T1 = 6% *Azolla filiculoides* meal; T2 = 10% *Azolla filiculoides* meal; T3 = 16% *Azolla filiculoides* meal; T4 = 23% *Azolla filiculoides* meal; n = Number of samples

All data are means ± standard error of the mean (S.E.M.)

[a,b,c] Mean with different superscripts within a row are significantly different ($p$<0.05)

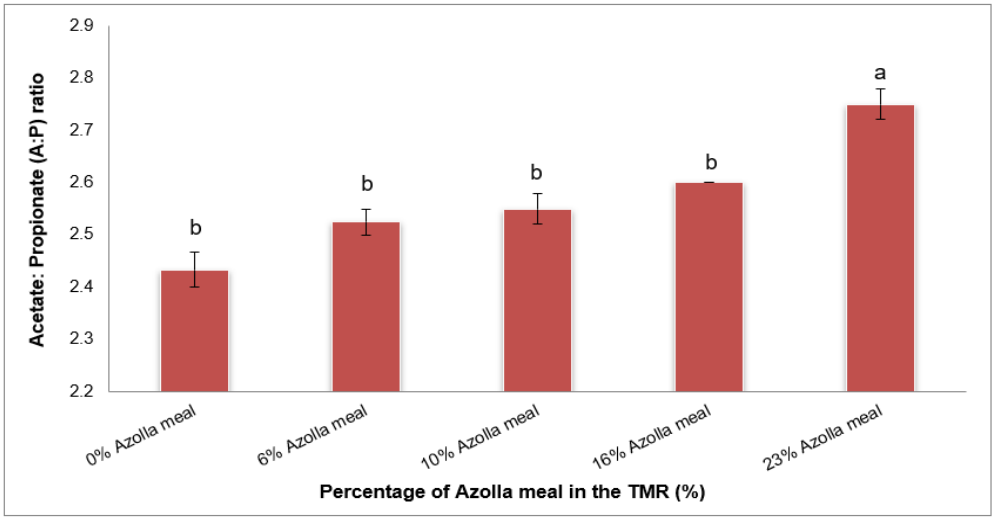


*Figure 2*. Acetic: propionic ratio of Napier silage total mixed ration with different inclusion percentage of *Azolla filiculoides* meal. All data are means ± standard error of the mean (S.E.M).
[a,b] Mean with different superscripts are significantly different ($p$<0.05)

## DISCUSSION

Inclusion of *A. filiculoides* meal at a rate of 6%–23% was able to retain the CP content at an average value of 15.0%CP kg$^{-1}$ DM after reducing soybean meal components at the range of 1.0% to 4.0%. It can be attributed to the *Azolla*'s high CP content, consisting of CP at the range of 19.4%–24.5%. Therefore, Kamaruddin et al. (2019) verified that this species was suitable for animal feed. According to Mohammad Fitri Rimi et al. (2021), *A. filiculoides* could be cultivated by fully utilizing the organic source such as manure from the livestock waste as their nutrient supplier for growth. A significant difference has occurred in this plant's biomass production and nutrient composition depending on the source and type of manure. Thus, the protein composition produced by *A. filiculoides* meal was adequate for ruminant requirement (Freer, 2007; NRC, 1996). However, the inclusion higher than 16% had affected the fiber and fat composition of Napier silage TMR. The highest CF values were obtained once replacing 19.0% Napier silage and 4.0% soybean meal with 23% *A. filiculoides* meal inclusion within the basal diet. As a result, it had showed an increment of 11.4% compared to control (0% *A. filiculoides* meal). However, it was lower than the CF of Napier grass forage between 33.0%–35.0% kg$^{-1}$ DM (Haryani et al., 2018). The CF of T1, T2, T3, and T4 was lower than the CF of rice straw total mixed rations, which reached 43.3% CF kg-$^{1}$ DM (Sarker et al., 2018). Therefore, it has been an indication of the suitability of this species as an alternative source of fiber for ruminants. Besides, CF produced through the inclusions was higher

than 15.4%, which is the optimum value to ensure an optimum acetic: propionic ratio produced at 3.0 or for the methane gas production below 6.9% MJ day$^{-1}$ (Luthfi et al. 2018).

Simultaneously, the inclusion of 10% *A. filiculoides* (T2) had consequent an increment of NDF at 8.7%, which had significantly declined 9.6% volume of 48 h cumulative *in vitro* gas production compared to the lower inclusion. The NDF value for *A. filiculoides*, which is 36.5%–37.6% kg$^{-1}$ DM (Mohammad Fitri Rimi et al., 2021), is lower than *Eichhornia crassipes* which is 65.9%–72.9% (Mako et al., 2011). Therefore, this species might enhance the feed intake of ruminants compared to other aquatic species. However, the high lignin composition of *A. filiculoides* (7.61%–9.02%) compared to *E. crassipes* and *Pistia stratiotes* (5.49% and 3.47% /kg DM) had slower digestibility once utilized at the higher percentage (Mani, 2019; Sivasankari & Ravindran, 2016).

A significant decrease could be seen in the cumulative *in vitro* gas production of TMR in line with higher inclusion than 6% into the ruminant diet. With this amount of inclusion, 157.6 mL g$^{-1}$ DM of cumulative *in vitro* gas production was recorded, and it was lower than 195.5 mL g$^{-1}$ DM, which was recorded from 40:60 TMR of sweet corn residue and rice straw (Kraiprom & Tumwasorn, 2017). However, as the cumulative *in vitro* gas production was inversely proportional with the percentage of the inclusion of *A. filiculoides* meal, Murillo-Ortiz et al. (2018) have reported a similar effect detected on the addition of *E. crassipes* in the alfalfa hay-based diet. However, the 48-h cumulative *in vitro* gas production of 23% inclusive had been recorded higher than the 209.0 mL g$^{-1}$ DM, which took from Zailan et al. (2016a)'s study on common Napier. It can be attributed to an increase in the value of the fiber component, which causes a higher duration for the degradation of fiber along the rumination process. The compositions of ADF and ADL for *A. filiculoides* plants were 27.6% and 7.61% were lower than *E. crassipes*, which was determined as 77.9% and 15.4% (Ganguly et al., 2013; Hossain et al., 2015; Mohammad Fitri Rimi et al., 2021). These factors have affected the catabolism process and nutrient absorption into an animal digestion system. With the 23% inclusion of *A. filiculoides* meal, the values of IVDMD and IVOMD were 41.2% and 23.0%, respectively. Even though those values were lower than the value obtained from the 25% *E. crassipes* with alfalfa hay reported by Murillo-Ortiz et al. (2018), they were higher than IVDMD and IVOMD of common Napier, which were measured at 54.6% and 50.8%, respectively. However, those values were still lower, and the ME reached 11.6% MJ kg$^{-1}$ DM compared to 7.3% MJ kg$^{-1}$ DM for common Napier (Zailan et al., 2016a). However, the ME value of T1 (6% *A. filiculoides*) was higher than the *Mucuna* bean (Castro et al., 2003).

The increment of *A. filiculoides* meal inclusion percentage had directly affected the concentration of total VFA at the range of 86.0–120.0 mM/L was in line

with the optimum range for total VFA for ruminant, which is between 80–120 mM/L as mentioned by McDonald et al. (2010). The high VFA in T1, T2, T3, and T4 was due to the degradation of cell wall components (NDF and ADF) into VFA, which was greater than control. The higher the level of fermentability of the feed ingredient, the greater the VFA produced other than those from protein because the VFA was derived from carbohydrates and protein. In this research, partial VFA for T3 was 64.2:16.2:15.6 for acetate, propionate, and butyrate, respectively. This ratio was near the proportion of good partial VFA ratio in the rumen, which Hungate (2013) stated, which is 63:21:16. Meanwhile, Jouany and Ushida (1999) also stated that the molar proportion in the rumen of various good feed formulations for acetate was 53–72 mM/L, while propionate was 15–30 mM/L and for butyrate was 7–21 mM/L. The tendency of a higher molar proportion of acetate in the treatment with higher inclusion of *A. filiculoides* meal indicates the potential for higher energy production for livestock diet to the higher ATP production in the substrate. As the percentage of acetate was increased, together with the percentage of *A. filiculoides* meal inclusion, the range of the A: P ratio increased from 2.2 to 3.0, as mentioned by Russell (1998).

## CONCLUSION

Based on this study, *Azolla filiculoides* meal was used in the ruminant diet as an alternative source of fiber and protein. Due to this species' low dry matter content, *A. filiculoides* could not be used as the main fodder for ruminants, especially cattle. However, this species was able to produce sufficient organic matter digested in the total ruminant digestive tract and will simultaneously affect the production of metabolizable energy for the animal. Furthermore, instead of using it in fresh form, this plant was more suitable to be used in the form of dried or meal. Inclusions of *A. filiculoides* meal at the level 6% to 10% in ruminant diets will help farmers enhance their productivity through their livestock performance by utilizing an alternative source of fiber and protein such as *A. filiculoides* meal. This plant was able to be used as the inclusion with the concentrate and Napier silage at 6% to replace 5% of Napier and 1% of soybean meal. A digestibility study should be conducted to determine the optimum inclusion of *A. filiculoides* between 6% to 10% in the TMR feed with or without fermentation treatment.

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## REFERENCES


Akbari, M., & Resalati, H. (2012). *Use of agricultural waste in the pulp and paper industry*. http://crowa.khuisf.ac.ir/DorsaPax/userfiles/file/pazhohesh/crowa91/61.pdf

Association of Official Analytical Chemists. (2005). *Official methods of analysis of the Association of Analytical Chemists*. AOAC.

Castro, C. S., Herrera, P., Leal, C. C., & Burgos, A. A. (2003). *In vitro* gas production and digestibility of *Mucuna* bean. *Tropical and Subtropical Agroecosystems*, *1*(2-3), 77-80.

Costa, M. L., Santos, M. C., & Carrapiço, F. (1999). Biomass characterization of *Azolla filiculoides* grown in natural ecosystems and wastewater. *Hydrobiologia*, *415*, 323-327. https://doi.org/10.1023/A:1003824426183

Cottyn, B. G., & Boucque, C. V. (1968). Rapid method for the gas-chromatographic determination of volatile fatty acids in rumen fluid. *Journal of Agricultural and Food Chemistry*, *16*(1), 105-107.

Devendra, C., Yeong, S. W., & Ong, H. K. (1983, December 14-15). *The potential value of palm oil mill effluent (POME) as a feed source for farm animals in Malaysia* [Paper presentation]. In National Workshop on Oil Palm By-Product Utilization. Session B: Feedstuff Production and Utilization, Kuala Lumpur, Malaysia

Escoto, D. F., Gayer, M. C., Bianchini, M. C., da Cruz Pereira, G., Roehrs, R., & Denardin, E. L. (2019). Use of *Pistia stratiotes* for phytoremediation of water resources contaminated by clomazone. *Chemosphere*, *227*, 299-304. https://doi.org/10.1016/j.chemosphere.2019.04.013

Freer, M. (Ed.). (2007). *Nutrient requirements of domesticated ruminants*. CSIRO Publishing.

Ganguly, A., Das, S., Bhattacharya, A., & Singh, P. (2013). Studies on the production of xylose from water hyacinth. *Advances in Chemical Science*, *2*(1), 1-7.

Haryani, H., Norlindawati, A. P., Norfadzrin, F., Aswanimiyuni, A., & Azman, A. (2018). Yield and nutritive values of six Napier (*Pennisetum purpureum*) cultivars at different cutting age. *Malaysian Journal of Veterinary Research*, *9*(2), 6-12.

Hossain, M. E., Sikder, H., Kabir, M. H., & Sarma, S. M. (2015). Nutritive value of water hyacinth (*Eichhornia crassipes*). *Online Journal of Animal and Feed Research*, *5*(2), 40-44.

Hungate, R. E. (2013). *The rumen and its microbes*. Elsevier.

Jouany, J. P., & Ushida, K. (1999). The role of protozoa in feed digestion - Review. *Asian-Australasian Journal of Animal Sciences*, *12*(1), 113-128. https://doi.org/10.5713/ajas.1999.113

Kamaruddin, N. A., Yusuf, N. M., Ishak, M. F., & Kamarudin, M. S. (2019). Study on chemical composition of *Azolla filiculoides* and *Hydrilla verticillata*. *Journal of Agrobiotechnology*, *10*(1S), 68-74.

Kollah, B., Patra, A. K., & Mohanty, S. R. (2016). Aquatic microphylla *Azolla*: A perspective paradigm for sustainable agriculture, environment and global climate change. *Environmental Science and Pollution Research*, *23*(5), 4358-4369. https://doi.org/10.1007/s11356-015-5857-9

Kraiprom, T., & Tumwasorn, S. (2017). Optimum proportion of sweet corn by-product silage (SCW) and rice straw in total mixed ration using *in vitro* gas production. *Agriculture and Natural Resources*, *51*(2), 79-83. https://doi.org/10.1016/j.anres.2016.10.007

Kum, W. H., & Zahari, M. W. (2011). Utilisation of oil palm by-products as ruminant feed in Malaysia. *Journal of Oil Palm Research*, *23*(1), 1029-1035.

Kumar, P. R., Sreelekshmi, K. S., Anjana, S. B., Harikrishnan, S., Santhanu, G. N., & Leena

V. P. (2020). Role of agricultural wastes in construction industry. *International Journal of Engineering Research and Technology*, *9*(3), 66-69. https://doi.org/10.17577/IJERTV9IS030127

Luthfi, N., Restitrisnani, V., & Umar, M. (2018). The optimation of crude fiber content of diet for fattening madura beef cattle to achieve good A:P ratio and low methane production. In *IOP Conference Series: Earth and Environmental Science* (Vol. 119, No. 1, p. 012056). IOP Publishing. https://doi.org/10.1088/1755-1315/119/1/012056

Mako, A. A., Babayemi, O. J., & Akinsoyinu, A. O. (2011). An evaluation of nutritive value of water hyacinth (*Eichhornia crassipes* Mart. Solms-Laubach) harvested from different water sources as animal feed. *Livestock Research for Rural Development*, *23*(5), 10.

Mani, A. M. M. (2019). Utilization leaf meal of water hyacinth (*Eichhornia crassipes*) as a replacement protein source for growing Awassi lambs. *International Journal of Veterinary Science*, *8*(1), 54-60.

McDonald, P., Edward, R. A, Greenhalgh, J. F. D., Morgan, C. A., Sinclair, L. A., & Wilkinson, R. G. (2010). *Animal nutrition* (7th ed.). Pearson-Prentice Hall.

Menke, K. H. (1988). Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Animal Research and Development*, *28*, 7-55.

Menke, K. H., Raab, L., Salewski, A., Steingass, H., Fritz, D., & Schneider, W. (1979). The estimation of the digestibility and metabolizable energy content of ruminant feeding stuffs from the gas production when they are incubated with rumen liquor *in vitro*. *The Journal of Agricultural Science*, *93*(1), 217-222. https://doi.org/10.1017/S0021859600086305

Mohammad Fitri Rimi, H., Muhammad Faisal, A. B., Mohd Hafizzudin, A., Habsah, B., Shohaimi, S., Noraini S., & Mohd Noor Hisham, M. N. (2021). Biomass production and nutritional composition of *Azolla filiculoides* cultivated in a different livestock manure. In *40th Malaysian Society of Animal Production Annual Conferences: Livestock Industries Surviving the Covid-19 Pandemic* (pp. 237-239). Malaysian Agricultural Research and Development Institute.

Murillo-Ortiz, M., Herrera-Torres, E., Corral-Luna, A., & Pamanes-Carrasco, G. (2018). Effect of inclusion of graded level of water hyacinth on *in vitro* gas production kinetics and chemical composition of alfalfa hay-based beef cattle diets. *Indian Journal of Animal Research*, *52*(9), 1298-1303.

National Research Council. (2001). *Nutrient requirements of dairy cattle: 2001*. National Academies Press.

Rosali, M. H. (2015). *The development and future direction of Malaysia's livestock industry*. https://ap.fftc.org.tw/article/960

Russell, J. B. (1998). The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production *in vitro*. *Journal of Dairy Science*, *81*(12), 3222-3230. https://doi.org/10.3168/jds.s0022-0302(98)75886-2

Sahota, A. (Ed.). (2014). *Sustainability: How the cosmetics industry is greening up*. John Wiley & Sons.

Sarker, N. R., Yeasmin, D., Tabassum, F., & Habib, M. A. (2018). Effect of paddy-straw based Total Mixed Ration (TMR) on milk yield, milk composition and rumen parameters in lactating Red Chittagong cows. *Bangladesh Journal of Livestock Research*, 69-81. https://doi.org/10.3329/bjlr.v0i0.45449

Seephueak, W., Ngampongsai, W., & Chanjula, P. (2011). Effects of palm oil sludge in concentrate on nutrient utilization and rumen ecology of Thai native cattle fed with hay. *Songklanakarin Journal of Science and Technology*, *33*(3), 271-280.

Shanmuganvelu, S. (2014). *Decision support system in livestock production*. Malaysian Agricultural Research and Development Institute.

Sivasankari, B., & Ravindran, D. (2016). A study on chemical analysis of water hyacinth (*Eichornia crassipes*), water lettuce (*Pistia stratiotes*). *International Journal of Innovative Research in Science, Engineering and Technology*, *5*(10), 17566-17570.

Theodorou, M. K., Williams, B. A., Dhanoa, M. S., McAllan, A. B., & France, J. (1994). A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Animal Feed Science and Technology*, *48*(3-4), 185-197. https://doi.org/10.1016/0377-8401(94)90171-6

Van Soest, P. J., Robertson J. B., & Lewis B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch carbohydrates in relation to animal nutrition. *Journal of Dairy Science*, *74*(10), 3583-3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Zailan, M. Z., Yaakub, H., & Jusoh, S. (2016a). *In vitro* digestibility and gas production characteristics of four Napier (*Pennisetum purpureum*) cultivars as fresh fodder. *Malaysian Journal of Animal Science*, *19*(2), 95-105.

Zailan, M. Z., Yaakub, H., & Jusoh, S. (2016b). Yield and nutritive value of four Napier (*Pennisetum purpureum*) cultivars at different harvesting ages. *American Journal of Agricultural and Biological Science*, *7*(5), 213-219.

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*Short Communication*

# Evaluation on Durian var. *Musang King* Pollination Compatibility Regarding High Fruit Set

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## ABSTRACT

Durian or *Durio zibethinus* of variety *Musang King* is growing in popularity and with high international demands. With the ever-increasing demands for fruits, growers are exploring ways to maximize production by looking at the feasibility of planting single or mono varieties in a planting area. Previous investigations revealed that many durian varieties are self-incompatible, and the condition varies from one variety to another. Against this background, the present study evaluated *Musang King*'s compatibility status in fruit sets. The study was conducted in Raub, Pahang, from 2017 through 2018 with five different pollination treatments. Crossing *Musang King* with D24 showed the highest fruit set rate of 16.28% at harvest and suggested this variety is self-incompatible. Observations on the flowering process revealed that *Musang King* possessed herkogamy condition, which posed a morphological barrier to self-pollination. The study proposes that *Musang King* is best planted in a multi-variety planting system instead of mono-variety to achieve a higher rate of fruit sets.

*Keywords*: Autogamy, herkogamy, *Musang King* variety, self-incompatibility, xenogamy

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## INTRODUCTION

In recent years, durian or *Durio zibethinus*, whose tree looks regal and majestic befitting its royal title as 'King of Fruits,' has become one of the most popular fruits for export by many Southeast Asian countries like Malaysia. In 2020 alone, Malaysia exported about 30,000 tonnes of the fruits valued at about RM74.1 million and has been expected to increase in the coming years leading to the

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establishment of more durian orchards with *Musang King* as the leading variety (Ahmad & Pfordten, 2021). Conventional planting of durian is by having a few varieties in a planting area with the main objective of getting high production capacity (Abidin et al.,1991). However, questions have arisen among growers about its viability in a monoculture cropping system. Thus, planting several varieties in a planting area has been recommended. It is being supported based on the occurrence of self-incompatibility among durian varieties. The ability to set fruits is associated with pollination, defined as the transfer of pollen (male gametophytes) to stigmas of female parts, which occurs in the same flower as a complete flower or another flower (Abrol, 2015). However, pollination is not always successful. It is due to the stigmas' ability to detect the genetic compatibility of the pollens, which dictates the eventual fertilization. Successful pollination leading to fertilization is indicated by fruit set. For that to happen, Sanzol and Herrero (2001) cited that an adequate quantity of pollen must be transferred to stigmas and consequent growth of pollen tubes takes place.

Variations in self-incompatibility (SI) within plants' families occur typically due to only one or few genes which control SI, which segregates self-incompatibility within the families for alleles at the gene(s) level. Lipow and Wyatt (1999) put forward that the pattern of inter-compatibility depends on the particular genetic system involved, which differs from inbreeding depression which is generally caused by many loci with no segregation. Assessing the plant's ability in terms of SI is important to understand the significant changes that occurred in the self-pollination avoidance system in angiosperms (Navarro et al., 2012). Lim and Luders (1997) published variabilities in the magnitude of SI among durian varieties studied, and in a separate report (Lim & Luders, 1998) stated that SI is cultivar dependent. Studies on Thai durian by Honsho et al. (2009) noted the existence of SI, although in earlier pollination studies. Honsho et al. (2004) stated that, in self-pollination tests, all self-pollinated durian showed low percentages of fruit sets except for variety *Kradum Thong* in which self-pollination exceeded the success rate recorded in cross-pollinated variety, *Phaung Manee*. Against this background, the variety *Musang King* held the potential of producing higher yield if self-pollination is enhanced, which could assist growers in deciding the planting system to be employed to maximize production.

The present study examined flower morphology in its contribution to the pollination habits of *Musang King*. The study aimed at investigating pollination compatibility of *Musang King* variety with respect to higher fruit settings by utilizing different pollen transfer procedures as treatments.

## MATERIALS AND METHODS

### Location

This study was conducted at Lembah Temir Resort, Lembah Klau, Raub Pahang (3.7182° N, 102.0347° E) from January

until December 2017 until 2018. The study location was at Raub, a popular durian town with its extensive cultivation of durian, especially the variety, *Musang King*. An orchard with two major varieties, *Musang King* and D24, bearing an age exceeding 20 years, was selected. The orchard had a history of being well-maintained with good farm management practices in fertilization, irrigation, and pest control carried throughout the cultivation of the crop.

**Plant Materials**

At the commencement of the study, flowers were tagged *in situ* from the bud initiation stage. Flower blooming timelines were recorded (Figure 1) to establish when the flowers were fully open and to identify the gaps in time between the full-bloom state and the beginning of anthers' dehiscence. The observation could assist in understanding pollination ecology and determining the most suitable time to initiate pollination treatments. Prior to treatments, pollens were sourced from freshly dehisced flowers from the varieties grown within the experimental location. *Musang King* was the maternal flower, while the paternal or pollen donors were from *Musang King* for self-pollination and variety D24 for cross-pollination treatments depending on the fresh availability of pollens in the orchard. Only D24 was used in this study as it is the only other variety accessible and reported available with flowers bearing in the Lembah Temir Resort besides *Musang King.* The timing of flowering was simultaneous with *Musang King* flowering period. Therefore, only available varieties in the same location were selected to preserve the freshness and viability of the pollen used in this study. Flower clusters were thinned out to make 10–12 cm gaps between clusters to reduce flower density and competition.

**Pollination Compatibility Test**

For the compatibility test, each flower cluster was treated as one replication. According to the pollen sources, five pollination treatments were used to pollinate the maternal flowers (*Musang King*). Treatments consisted of the following:

i) Self-pollination treatment with pollens of *Musang King* from the same tree (PST)
ii) Cross-pollination treatment within variety where pollens of *Musang King* sourced from different trees were used (PDT)
iii) Autonomous autogamy pollination treatment where *Musang King* flowers were left untouched, no thinning and no emasculation but covered with plastic bags (autogamy)
iv) Pollination treatment with D24 pollens (xenogamy), and
v) Open-pollination treatment where flower clusters were tagged without alteration or modification (control).

Flower clusters in all treatments, except open pollination, were wrapped in plastic bags for seven days before and after anthesis (DAA) to eliminate contamination and visitation by other

visitors. All anthers of flowers on treatment plants were emasculated at noon before the flowers were fully open, and all flower clusters of treatment plants were thinned out, leaving only seven to 12 flowers per cluster, except for autonomous autogamy and open-pollination treatments. All flowers for PST, PDT, and xenogamy treatments were pollinated by hand pollination or assisted pollination. Flowers were pollinated with freshly dehisced pollens collected late evening and re-wrapped with plastic bags after treatments. All parts of the stigmas were fully covered with fresh pollens to ensure sufficient pollens were applied to stigmas. Each flower cluster used in this experiment was considered a replicate. Pollination treatments were performed on 12 flower clusters of *Musang King* for each pollination treatment (n = 60).

**Pollen Tube Observation**

In the procedure, ovaries of treated flowers were cut-off from pistil samples, and the outer layers of the ovaries were excised to expose the ovules. Samples were collected three days after anthesis and stored in a formaldehyde alcohol acetic acid (FAA) fixative. Subsequently, the samples were softened using 8M sodium hydroxide (NaOH)for 14 days in a 100 ml glass bottle. Next, the samples were clean-off from NaOH solution with distilled water before staining with aniline blue in 0.1M potassium phosphate ($K_3PO_4$) adjusted to an acidic pH 5. Overnight staining was allowed in the dark before placing the samples on microscope slides with drops of glycerol on the slides before covering the samples for observation. The samples were observed under fluorescence microscope Leica DFC310 FX (Germany) with excitation of 360 nm Filter 1. Procedures were modified from Kozai et al. (2014) and Bumrungsri et al. (2009) to suit this experiment.

**Data Collection**

Honsho et al. (2004) stated that many young fruits dropped two to eight weeks after pollination, and their data showed stability in fruit set (%) at eight days after pollination treatments, and before that showed the same pattern of decreased number of fruit set for all their pollination treatments. On the other hand, Kozai et al. (2014) study stated that the frequencies of deformed ovules among the treatments between three days and seven days do not significantly differ. In addition, according to Bumrungsri et al. (2009), the majority of the fruit set abortions happen within 20 days after pollination experiments, and it decreased after that period. Thus, the data collections began on the seventh day after the pollination date and continued at the $14^{th}$, $21^{st}$, $28^{th}$, and at harvest was suitable to portray the fruit set (%) pattern during the overall period from pollination to harvest.

**Statistical Analysis**

Pollination treatments on the fruit set were calculated as a percentage per cluster for each replication. Fruit sets were recorded on the $7^{th}$, $14^{th}$, $21^{st}$, and $28^{th}$ days after anthesis (DAA) and harvest day. The collected data were subjected for normality test using

diagnostic regression plot in SAS (version 9.4), and from a fit diagnostic graph, residual of data collected is normally distributed. In addition, data of fruit set (%) recorded were subjected to analysis of variance (ANOVA), and comparison of means was subjected to Tukey's range test.

## RESULTS AND DISCUSSION

### Flower Blooming and Anther Dehiscence

Figure 1 presents flowering timelines in the durian variety *Musang King*. The study observed that the epicalyx of a flower bud started to break a day before the flowers bloomed. The blooming of *Musang King* flowers could be seen as protrusions of flower buds in the morning and proceeded by an elongation of the corolla before the flowers started to open in late the afternoon. Blooming progressed until the petals were fully retracted, touching the calyx in the evening at around 6.30 p.m. and exposing the stigmas and stamens.

In anther dehiscence, pollens were observed to consistently release pollens only around 7.30 p.m. when the sun had already set. The release of pollens started with the break of stomium. At the beginning of the release, pollens were observed to be dry and subsequently seen to become wet after an hour. Salakpetch et al. (1991) recorded that the round-shape durian pollen grains appeared sticky and released in clumps. Sanchez et al. (2004) reported that this sticky condition of the pollen combined with stigma exudate, which contained both proteins and sugars, helped in the adhesion of pollens. Due to this stickiness of the pollens, pollen transfers were possible, without which, and without the help of a pollinator, were reported to be impossible (Bumrungsri et al., 2009). Shivanna and Tandon (2014), in their studies, reported that there were time gaps of about three hours and 30 minutes between the time when the stigmas started to be exposed (which was the time when the flower buds started to open at 4 p.m.) and time when anthers released the pollens (7.30 p.m.) making a condition known as protogyny (where stigmas became receptive before the pollens started to function).

### Pollination Compatibility

The percentages of fruit sets after anthesis and pollination treatments and after harvest are presented in Table 1. Treatment with pollens from different *Musang King* trees (PDT) recorded a higher rate of fruit set

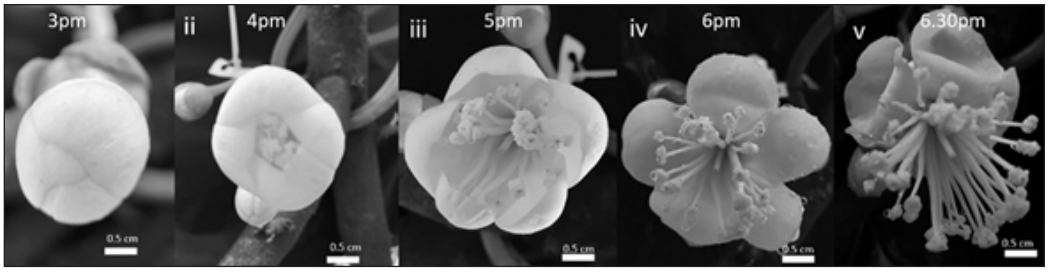


*Figure 1*. Flowering Timeline in *Musang King* durian

Table 1
*Percentages of fruit sets at days after anthesis*

| Treatment | Days after anthesis (DAA) | | | | |
|---|---|---|---|---|---|
| | 7th | 14th | 21st | 28th | Harvest |
| Control | 22.36$^{ab*}$ | 3.49$^{b}$ | 3.49$^{b}$ | 2.05$^{b}$ | 0.87$^{b}$ |
| PDT | 37.15$^{a}$ | 0$^{b}$ | 0$^{b}$ | 0$^{b}$ | 0$^{b}$ |
| PST | 9.27$^{b}$ | 0.85$^{b}$ | 0$^{b}$ | 0$^{b}$ | 0$^{b}$ |
| Xenogamy | 20.79$^{ab}$ | 16.28$^{a}$ | 16.28$^{a}$ | 16.28$^{a}$ | 16.28$^{a}$ |
| Autogamy | 5.2$^{b}$ | 0$^{b}$ | 0$^{b}$ | 0$^{b}$ | 0$^{b}$ |

*Note*. *Means with the same letter vertically are not significantly different at $P \leq 0.05$ using the Tukey test.
DAA: Days after anthesis; PDT: Pollination from different trees; PST: Pollination from the same tree

(31.15%) compared to pollination with pollen from the same tree (PST) recorded at 9.27%. Autonomous autogamy pollination showed a significantly low fruit set at 5.2%, whereas control or open pollination yielded 22.36%, and xenogamy resulted in a 20.79% fruit set. Fruit set for all treatments continuously dropped except for xenogamy in which fruit set stable started from 14 days after pollination or day after anthesis (DAA) and consistently maintained at 16.28% until harvest time. Open pollination (control) recorded 0.87% fruit set at harvest. The data suggest that on day 14$^{th}$ after anthesis, the fruit sets were stable and could be used as an indicator in predicting fruit production if the appropriate pollination procedure was carried out. The significant difference in fruit sets for control (open pollination) and xenogamy (*Musang King* crossed with D24) gave an insight into the importance of not only pollen load and availability and pollens' compatibility to yield high fruit sets. In similar studies on durian, Bumrungsri et al. (2009), Honsho et al. (2004, 2007) reported that the percentages of fruit sets were generally the lowest for open pollination, followed by self-pollination, while assisted pollination was recorded higher fruit sets.

The control (open pollination) recorded a significantly higher percentage of fruit sets. Similar responses were recorded with xenogamy on the 7$^{th}$ day after anthesis, but the percentage was significantly lower on the 14$^{th}$ day as it dropped to 3.49%. In autogamy, pollination had resulted in a significantly low percentage of fruit set on the seventh day after anthesis. No fruit set was recorded on the 14$^{th}$ day after anthesis. The significantly low fruit set rate in treatment by autogamy could be due to absence or very low pollen load. Wilcock and Neiland (2002) reported that the number of pollens transferred during assisted pollination had significant effects on pollination success as insufficient pollens quantity caused a low number of ovules being fertilized and resulted in low fruit sets. In the present study, assisted cross-pollination of *Musang King* and D24 yielded confirmed high fruit sets starting on the 14$^{th}$ day after anthesis compared to other pollination treatments suggesting that assisted cross-pollination had a higher rate

for fruit set in comparison with assisted self-pollination treatment.

Wilcock and Neiland (2002) cited that one of the reasons for pollination failure in plants was insufficient pollens, which resulted in a low number of ovules compared to the total number of ovules being fertilized, thus negatively impacting stimulation for fruits to set. Data on treatments by PDT and PST, which yielded 0% of fruit set on the 21st day after anthesis, proved no difference in reaction on compatibility when *Musang King* was pollinated within the variety. Kozai et al. (2014) studied ovule development in cross-pollinated and self-pollinated Thai durian cultivars and recorded that all non-pollinated flowers under the study had all ovules degenerated. About 82% degenerated ten days after anthesis (DAA), and on 14 days after anthesis (DAA), there were still 5% fruit sets suggesting that although there was no pollination that took place, the ovaries could set fruiting and remain on the tree for a period after anthesis. In the present study on *Musang King*, fruit setting in autogamy treatment on the 7th day after anthesis could be caused by the apomixis development but later by abortion significantly on the 14th day after anthesis. A similar phenomenon occurred in self-pollinated (PST) and cross-pollinated same variety (PDT) pollination treatments. The pistil from these treatments remained on the branches and dropped 14th day after anthesis or hand-pollination. The pollination compatibility test on *Musang King* confirmed self-incompatibility syndrome on the 21st day after pollination. There was 0% fruit set in self-pollinated (PST and PDT) treatments.

Results of cross-pollination between *Musang King* and D24 in xenogamy treatments agreed with previous studies of Bumrungsri et al. (2009), Honsho et al. (2004, 2007), who reported high fruit sets from cross-pollination of different varieties of durian. The ability to yield higher fruit sets in cross-pollination instead of self-pollination was caused by self-incompatibility (Honsho et al., 2004). Self-incompatibility in the Bombacaceae family in which *Durio zibethinus* belongs, have been discussed in several species such as *Eriotheca gracilipes*, *Ceiba petandra*, and *Theobroma cocoa*, many of which have self-incompatibility issues and have high fruit sets when cross-pollinated (Ford & Wilkinson, 2012; Gribel et al., 1999; Oliveira et al., 1992). The possibility of self-incompatibility to cluster within family and close families was discussed by Gibbs and Bianchi (1999), where the heredity of a single locus established by the SI mechanism could have been passed down within the family. From flower blooming stages as presented in Figure 1, the *Musang King*'s flowers at full bloom have their stigmas and anthers in spatial separation. It was observed that the flowers have protogyny conditions as the stigmas were exposed earlier than the anthers. The spatial separation between the anthers and stigmas showed that *Musang King*'s flowers have herkogamy conditions. Previous studies by Lim and Luders (1997) cited that at anthesis, the stamens and stigmas had

the same height but did not elaborate the conditions to the effect on self-pollination ability. Webb and Llyod (1986) reported that many self-incompatible plants possessed herkogamy conditions which could be the reason for failed self-pollination. Luijten et al. (1999) discussed herkogamy conditions and suggested reducing risk using pollen from anthers of the same flower.-

Reduction of self-fertilization had been reported for species *Gentianella germanica* and *Narcissus cyclamineus* (Luijten et al., 1999; Navarro et al., 2012) caused by herkogamy. In a study on *Habranthus gracilifolius*, Streher et al. (2018) reported that herkogamy was a barrier to self-pollination and self-incompatibility. They concluded that both herkogamy and self-incompatibility were a pre-and post-barrier of self-pollination and self-incompatibility. In a study on durian variety *Mon Thong*, Honsho et al. (2004) mentioned heterostyly, a reciprocal herkogamy where distyly or tristyly exist in a population (Jesson, 2017); however, approached herkogamy condition was consistently observed on all flowers of durian variety *Musang King* with a height of stigma exceeds the height of the anthers with spatial separation. Distyly or tristyly conditions were not observed from samples of *Musang King* flowers. Webb & Llyod (1986) had classified different types of herkogamy with different families classified under it, which means the herkogamy condition could be fixed as a morphological trait within the family. Despite the failure to retain fruit set after 14$^{th}$-day anthesis (DAA) as seen in Table 1, pollens were successfully grown into the micropyles as seen in Figure 2 for PDT pollination treatment. It indicates that *Musang King* could grow the pollen tube, and the termination happens in the ovule as in late acting self-incompatibility.

The success of pollen tubes of self-pollinated to grow in incompatible ovules suggests gametophytic self-incompatibility (Golz et al.,1995; Takayama & Isogai, 2005). Another plant species that exhibited incompatibility through tests of cross and self pollinations was *Lycium cestroides*. In pollination treatments of self-cross, geitonomous, autogamous, autonomous, and control treatment, Aguilar and Bernadello (2001) recorded that only cross-and open-pollination yielded fruits. On the other hand, self-and geitonomous hand-pollination and autonomous self-pollination were observed to have successful growth of pollen tubes in the ovules. Therefore, the authors concluded that the plant species had ovarian self-incompatibility or late-acting self-incompatibility conditions (Aguilar & Bernadello, 2001).

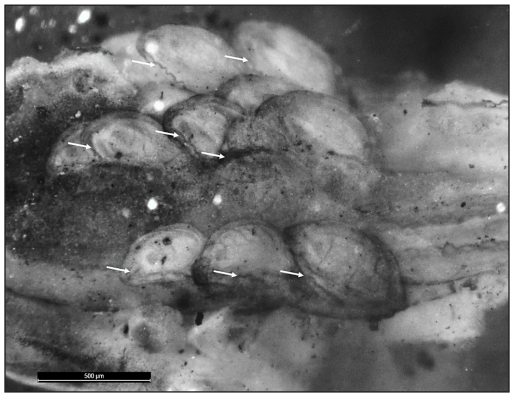


*Figure 2*. The pollen tubes grow in micropyles in PDT treatment with arrows pointing to the pollen tubes. ×100 scale bar = 500µm

Literature has it that the self-incompatibility system is divided into three types: Solanaceae, Papaveraceae, and Brassicaceae systems. The Solanaceae system acts by blocking growth incompatible pollen tubes growth in the pistil by the reaction of multi-allelic RNase. In contrast, the Papaveracea system acts by building calcium fluxes, actin rearrangements, and occurrence of cell death once the incompatible pollens were detected as a reaction from complex multicellular responses. The activation of the receptor kinase signaling pathway in the pistil to reject pollen is how the Brassicaceae system works (Silva & Goring, 2001). In the case of *Musang King*, it was not feasible to differentiate if the self-incompatibility system was one of the categories of self-incompatibility as the present study observed the ability of pollen tubes to grow in the micropyle of PDT and PST treatments. Furthermore, Kozai et al. (2014) recorded the occurrence of abortion after fruit set. Further investigation on the type of self-incompatibility system in *Musang King* would be useful for breeding purposes in the future.

## CONCLUSION

Failure of autogamy in the present study suggests that *Musang King* was unable to set fruits by apomixis without the help of a pollinator agent. Failures in PST and PDT treatments suggest that *Musang King* could not produce yield by its pollens. Ruling out of autogamy and geitonogamy, the only option left in the breeding system for high fruit set and high fruit production at harvest in *Musang King* was xenogamy compared to open pollination. Herkogamy, which exists in the flower morphology of *Musang King,* explains the reduced potential for self-pollination, as well as an important morphological marker to analyze the plant's ability to self-pollinate. An extensive study on flower morphology of other durian varieties should be carried out to enlighten us further on the pollination pattern and relation to self-incompatibility in durian species. Examination of the pollen tubes and their ability to set fruit compared to different pollens used either originated from *Musang King* or other durian variety reflects the capability of self-fertilized or *vice versa*. Low fruit-set percentages after self-pollination confirmed the self-incompatibility status of *Musang King*, and it should be planted with other varieties in a planting area. Results from the present study could guide growers of *Musang King* to decide on the implementation of a multi-varieties planting system instead of mono-variety. Although the multi-variety system could raise the number of trees to produce more fruits, it solely could not ensure pollination success. Compatible pollens, the existence of pollinator agents, and quality pollens should co-exist or simultaneously improve to increase durian fruit production. Further evaluation on different potential pollen donors could be done to examine crossing capabilities with *Musang King* as maternal to produce the highest number of fruits at harvest.

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## REFERENCES

Abidin, M. Z., Tarmizi, S. A., & Azizar, O. (1991). *Penanaman durian* [Planting of durian]. Malaysia Agriculture Research and Development Institute.

Aguilar, R., & Bernardello, G. (2001). The breeding system of *Lycium cestroides*: A Solanaceae with ovarian self-incompatibility. *Sexual Plant Reproduction*, *13*(5), 273-277. https://doi.org/10.1007/s004970100068

Ahmad, R., & Pfordten, D. (2021, July 18). Interactive: Durian season is in full swing, and here's what you need to know. *The Star*. https://www.thestar.com.my/news/nation/2021/07/18/interactive-durian-season-is-in-full-swing-and-here039s-what-you-need-to-know

Bumrungsri, S., Sripaoraya, E., Chongsiri, T., Sridith, K., & Racey, P. A. (2009). The pollination ecology of durian (*Durio zibethinus*, Bombacaceae) in southern Thailand. *Journal of Tropical Ecology*, *25*(01), 85-92. https://doi.org/10.1017/S0266467408005531

Ford, C. S., & Wilkinson, M. J. (2012). Confocal observations of late-acting self-incompatibility in *Theobroma cacao* L. *Sexual Plant Reproduction*, *25*(3), 169-183. https://doi.org/10.1007/s00497-012-0188-1

Gibbs, P. E., & Bianchi, M. B. (1999). Does late acting self-incompatibility (LSI) show family clustering? Two more species of Bignoniaceae with LSI: *Dolichandra cynanchoides* and *Tabebuia nodosa*. *Annals of Botany*, *84*(4), 449-457. https://doi.org/10.1006/anbo.1999.0933

Golz, J. F., Clarke, A. E., & Newbigin, E. (1995). Self-incompatibility in flowering plants. *Current Opinion in Genetics and Development*, *5*(5), 640-645. https://doi.org/10.1016/0959-437X(95)80033-6

Gribel, R., Gibbs, P. E., & Queiroz, A. L. (1999). Flowering phenology and pollination biology of *Ceiba pentandra* (Bombacaceae) in Central Amazonia. *Journal of Tropical Ecology*, *15*(3), 247-263. https://doi.org/10.1017/S0266467499000796

Honsho, C., Somsri, S., Salakpetch, S., Tetsumura, T., Yonemoto, Y., & Yonemori, K. (2009). Pollen sources effects on seed formation and fruit characteristics in Thai durians. *Tropical Agriculture and Development*, *53*(1), 28-32. https://doi.org/10.11248/jsta.53.28

Honsho, C., Somsri, S., Tetsumura, T., Yamashita, K., & Yonemori, K. (2007). Effective pollination period in durian (*Durio zibethinus* Murr.) and the factors regulating it. *Scientia Horticulturae*, *111*(2), 193-196. https://doi.org/10.1016/j.scienta.2006.10.016

Honsho, C., Yonemori, K., Somsri, S., Subhadrabandhu, S., & Sugiura, A. (2004). Marked improvement of fruit set in Thai durian by artificial cross-pollination. *Scientia Horticulturae*, *101*(4), 399-406. https://doi.org/10.1016/j.scienta.2003.11.019

Jesson, L. (2017). Reproductive strategies. In B. Thomas, B. Murray, & D. J. Murphy (Eds.), *Encyclopedia of applied plant science*, (2nd ed., pp. 321-326). Academic Press. https://doi.org/10.1016/B978-0-12-394807-6.00045-9

Kozai, N., Chusri, O., Chutinanthakun, T., Tongtao, S., Higuchi, H., & Ogata, T. (2014). Pollination and subsequent ovule development through fruit

set in 'Chanee', 'Monthong', and 'Kradumthong' durian. *Tropical Agriculture and Development*, *58*(2), 58-65. https://doi.org/10.11248/jsta.58.58

Lipow, S. R., & Wyatt, R. (1999). Floral morphology and late-acting self-incompatibility in *Apocynum cannabinum* (Apocynaceae). *Plant Systematics and Evolution*, *219*(1-2), 99-109. https://doi.org/10.1007/BF01090302

Lim, T. K., & Luders, L. (1997). *Boosting durian productivity*. Rural Industries Research and Development Corporation.

Lim, T. K., & Luders, L. (1998). Durian flowering, pollination and incompatibility studies. *Annals of Applied Biology*, *132*(1), 151-165. https://doi.org/10.1111/j.1744-7348.1998.tb05192.x

Luijten, S. H., Oostermeijer, J. G. B., Ellis-Adam, A. C., & den Nijs, J. H. C. (1999). Variable herkogamy and autofertility in marginal populations of *Gentianella germanica* in the Netherlands. *Folia Geobotanica*, *34*(4), 483. https://doi.org/10.1007/BF02914924

Navarro, L., Ayensa, G., Ferrero, V., & Sánchez, J. M. (2012). The avoidance of self-interference in the endemic daffodil *Narcissus cyclamineus* (Amaryllidaceae). *Plant Ecology*, *213*(11), 1813-1822. https://doi.org/10.1007/s11258-012-0137-y

Oliveira, P. E., Gibbs, P. E., Barbosa, A. A., & Talavera, S. (1992). Contrasting breeding systems in two *Eriotheca* (Bombacaceae) species of the Brazilian cerrados. *Plant Systematics and Evolution*, *179*(3-4), 207-219. https://doi.org/10.1007/BF00937597

Salakpetch, S., Chandraparnik, S., & Hiranpradit, H. (1991). Pollen grains and pollination in durian, *Durio zibethinus* Murr. *Acta Horticulturae*, *321*, 636-640. https://doi.org/10.17660/ActaHortic.1992.321.76

Sanchez, A. M., Bosch, M., Bots, M., Nieuwland, J., Feron, R., & Mariani, C. (2004). Pistil factors controlling pollination. *The Plant Cell*, *16*(Suppl. 1), S98-S106. https://doi.org/10.1105/tpc.017806

Sanzol, J., & Herrero, M. (2001). The "effective pollination period'' in fruit trees. *Scientia Horticulturae*, *90*(1-2), 1-17. https://doi.org/10.1016/S0304-4238(00)00252-1

Shivanna, K. R., & Tandon, R. (2014). Seedling recruitment. In *Reproductive ecology of flowering plants: A manual* (pp. 145-162). Springer. https://doi.org/10.1007/978-81-322-2003-9_12

Silva, N. F., & Goring, D. R. (2001). Mechanisms of self-incompatibility in flowering plants. *Cellular and Molecular Life Sciences*, *58*(14), 1988-2007. https://doi.org/10.1007/PL00000832

Streher, N. S., Guerra, E., Lüdtke, R., Semir, J., & Dutilh, J. H. A. (2018). Self-incompatibility in *Habranthus gracilifolius* (Amaryllidaceae): Pre- and post-pollination barriers. *Brazilian Journal of Botany*, *41*(2), 375-384. https://doi.org/10.1007/s40415-018-0463-y

Takayama, S., & Isogai, A. (2005). Self-incompatibility in plants. *Annual Review of Plant Biology*, *56*(1), 467-489. https://doi.org/10.1146/annurev.arplant.56.032604.144249

Webb, C. J., & Lloyd, D. G. (1986). The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy. *New Zealand Journal of Botany*, *24*(1), 163-178. https://doi.org/10.1080/002882 5X.1986.10409726

Wilcock, C., & Neiland, R. (2002). Pollination failure in plants: Why it happens and when it matters. *Trends in Plant Science*, *7*(6), 270-277. https://doi.org/10.1016/S1360-1385(02)02258-6

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# Effect of Sandwich Compost Leachate on *Allium tuberosum* Seed Germination

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## ABSTRACT

Food waste is a serious global issue, and one way to reduce the impact of food waste is by composting. Sandwich compost is a type of fermented food waste compost created with microbial fermentation; meanwhile, the composting leachate provides nutrients for plants. Studies have shown that seed germination may be enhanced when treated with sandwich compost leachate. Furthermore, few studies have been on sandwich compost leachate used for seed priming. The objective of this study was to determine the effect of varying leachate concentrations of food waste sandwich compost and priming durations on the performance of Chinese chive (*Allium tuberosum*) seed germination. Chinese chive (*Allium tuberosum*) was chosen as the test crop. It is widely used as a flavouring herb with high economic potential; however, its seed germination time is long and requires pre-treatment such as crushing and seed priming to speed up the germination process. The study used four replications and a complete randomisation design (CRD). The seeds were exposed to different percentages of sandwich compost leachate (0.0%, 0.2%, 0.4%, 0.6%, 0.8%, and 1.0%) and priming duration (4, 8, and 12 hours). A significant interaction between the bio-nutri-priming concentration and priming duration was demonstrated by measuring the standard error of germination rate ($S_{\overline{V}}$) and corrected germination rate index ($S_{corrected}$). A longer bio-nutri-priming duration was key for a higher seed vigour index. The bio-nutri-priming concentration and priming duration, however, had no significant interaction.

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Longer bio-nutri-priming durations were recommended to obtain better germination performance of Chinese chive. The study showed that a twelve-hour bio-nutri-priming duration and a 0.6 % leachate concentration significantly enhanced the Chinese chive seed germination and helped break seed dormancy.

*Keywords*: Bio-nutri-priming, Bokashi, Chinese chive, kucai, seed germination, seedling vigour index

## INTRODUCTION

Food waste is a serious problem around the globe. Hence, food waste utilisation is vital to reduce the environmental impact of food waste. Most people are not vegetarian; thus, composting methods that accept meat and dairy compost is crucial. The sandwich compost method utilises meat and dairy waste products without attracting pests at home. This form of food waste management could extend landfill life. Furthermore, leachate derived from food waste sandwich compost is considered an eco-friendly source by recycling nutrients for food production. The use of food waste in seed priming as the raw material meets the United Nations Sustainable Development Goals, including reducing poverty, hunger, and sustainable consumption.

Seed priming is a common solution to improve seed germination performance. Priming is an adjustment of water potential, which allows for seed imbibition but prevents germination. Biopriming, a mix of beneficial microbes and bioactive molecules, is associated with endophytic connections between flora and specific microbial. Biopriming is a sustainable method to support plant growth and development (Toribio et al., 2021). For instance, phytohormones production, abiotic and biotic stress resistance, and germination performance were enhanced by biopriming (Makhaye et al., 2021; Moeinzadeh et al., 2010; Paparella et al., 2015). Biopriming has significantly enhanced seed germination and plant growth performance of bread, wheat, and sunflower (Liela et al., 2010; Moeinzadeh et al., 2010).

Nutrient seed priming with molybdenum, zinc, boron, and phosphate was widely studied in Asian countries such as India, Nepal, Pakistan, and Bangladesh (Harris et al., 2001). Nutrient seed priming enhanced nutrient-use efficiency, photosynthetic rates, and translocation of reserves in an integrated manner (Davis & Quick, 1998). Surprisingly, the wheat yield increased up to 36% (Harris et al., 2001). Nutrient seed priming not only showed a positive effect in wheat seeds but also in corn seeds (Harris et al., 2001; Imran et al., 2013; Rasool et al., 2019), barley (Ajouri et al., 2004), and mung beans (Shah et al., 2012). Micronutrient seed priming also significantly enhanced the tolerance of corn to abiotic stress like salinity (Imran et al., 2018).

Therefore, the approach of bio-nutri-priming could shorten the priming duration with different concentrations of the leachate. Biopriming showed a positive effect on seed germination performance, particularly sandwich compost leachate (Bisen et al., 2015). Sandwich compost leachate is the by-product of fermented composting, resulting

in nutritive liquid leachate enriched with fermentative microbial. Therefore, sandwich compost leachate has the potential to improve seed germination performance by biopriming. The biopriming duration and leachate concentration are crucial in seed biopriming with sandwich compost leachate. Biopriming with sandwich compost leachate increased plant nutrient uptake and enhanced the stem diameter of tomato transplants by up to 13% (Olle, 2020).

Chinese chives (*Allium tuberosum*) were used as a test crop in this study. They are a widely used allium with aromatic flavoured leaves (sulphur-containing compounds) (Wang et al., 2008). Chinese chives have many health benefits, such as anti-diabetic and hepatoprotective properties (Tang et al., 2017). In addition, they are produced vegetatively with nonedible storage rhizome normally (Kamenetsky & Rabinowitch, 2017). Therefore, propagating the seed can increase genetic diversity. Nonetheless, the seed germination period is long, generally between 7 to 14 days. Usually, the seeds are primed between 12 to 24 hours to enhance germination. However, despite undergoing the priming process using different solutions, a longer priming period was required to enhance the germination performance. For instance, Chinese chives primed with 100 mg $L^{-1}$ of gibberellin for 12 to 24 hours showed a higher germination performance (Sun et al., 2010).

Thus, the objective of this study was to determine the effect of varying leachate concentrations of food waste sandwich compost and priming durations on the performance of Chinese chive (*Allium tuberosum*) seed germination.

## MATERIALS AND METHODS

### Treatments and Experiment Design

The experiment was carried out at Universiti Putra Malaysia (UPM) with coordinates 2°59'34.0"N, 101°42'52.3"E. The treatments consisted of six varying concentration percentages of sandwich compost leachate (0%, 0.2%, 0.4%, 0.6%, 0.8%, and 1.0%) (Table 1), and three priming durations (4, 8, and 12 hours). Seventy-two experimental units were arranged using a completely randomised design (CRD) with four replications. Each replication consisted of 30 seeds. At room temperature, the seeds were germinated in diameter (Ø) a 9 cm petri dish with two layers of moist tissue paper. The experiment was conducted at 27 ± 1°C under a 16:8 h light/dark photoperiod. The tissue paper was kept moist by spraying tap water every four hours.

Germinated seeds were counted daily until day ten. Then, daily counts of seedlings during the germination test were performed, whereby the seeds were considered to have germinated when there was visible radicle protrusion of at least 0.2 cm. Germination traits were calculated using the germination metrics package run in the R-program statistical software, which includes germination percentage (GP), standard error of germination rate ($S_{\overline{V}}$), germination rate as the reciprocal of the median time ($V_{50}$), corrected germination rate index ($S_{corrected}$), germination index (GI), and peak value (PV) (Aravind et al., 2019).

Table 1
*Physiochemical parameter of tap water and sandwich compost leachate*

| Physiochemical parameter | Tap water | Sandwich compost leachate |
|---|---|---|
| pH | 6.98±0.02* | 4.78±0.011 |
| Electric conductivity (dS $m^{-1}$) | 0.134±0.00 | 0.3357±0.0003 |
| Total dissolved salt (mg $L^{-1}$) | 85.76±0.00 | 22.19±0.2133 |
| Osmotic potential (bar) | 0.04824±0.00 | 0.0125±0.0001 |
| Total N (%) (Distillation and titration) | 0.00056±1.63×$10^{-18}$ | 0.2135±0.0052 |
| Phosphorous (ppm) | Not detected | 5833±223 |
| Potassium (ppm) | 3.64±0.0415 | 3941±131 |
| Calcium (ppm) | 13.8±0.150 | 528±18.6 |
| Sodium (ppm) | Not detected | 332±151 |
| Manganese (ppm) | Not detected | 72.0±2.67 |
| Iron (ppm) | 0.306±0.015 | 160±42.5 |
| Zinc (ppm) | Not detected | 161±7.51 |

*Note*. *mean ± standard error

## Seedling Vigour Index

ImageJ (Fuji, Japan) was used to analyse the root and shoot length at day ten. Seedling vigour is the total sum of seed properties that determine the seed or seed lot's level of activity and performance during seed germination and seedling emergence (International Seed Testing Association [ISTA], 1995). Low seed vigour means the seeds cannot perform all the physiological functions that allow them to germinate (ISTA, 1995). The seedling vigour index of the 10-day-old seedlings was calculated using the equation: root length + shoot length × germination percentage (%).

## Sandwich Compost Leachate Preparation

The sandwich compost preparation method was modified according to Christel (2017) and Phooi et al. (2021) (Figure 1). Effective microorganisms (EM) were used to prepare the sandwich taster. EM contains a larger number of lactic acid bacteria and yeasts and a minor quantity of phototrophic bacteria, filamentous fungi, and actinomycetes in a pH 3.5 liquid culture (Higa, 2001; Higa & Parr, 1994). An initial mixture was made with one part EM and one part of molasses dissolved in 45 parts of water. Next, the sandwich taster was prepared with one part of the mixture mixed with two parts of rice bran. The taster was kept in an opaque garbage bin and covered with a black garbage bag for two weeks before sun-dried. The sandwich compost bucket was self-made using two garbage bins: the upper bin with 26 holes (⌀2 mm in size) drilled at the bottom and the lower bin with a tap. The ratio of 3:2 of cropped 2 cm collected raw and cooked plant and animal-based food waste were layered in the bin. The waste mixture was alternately layered and compacted with 1 cm of sandwich taster and 5 cm of food waste. The leachate was harvested on day 14 of fermentation.

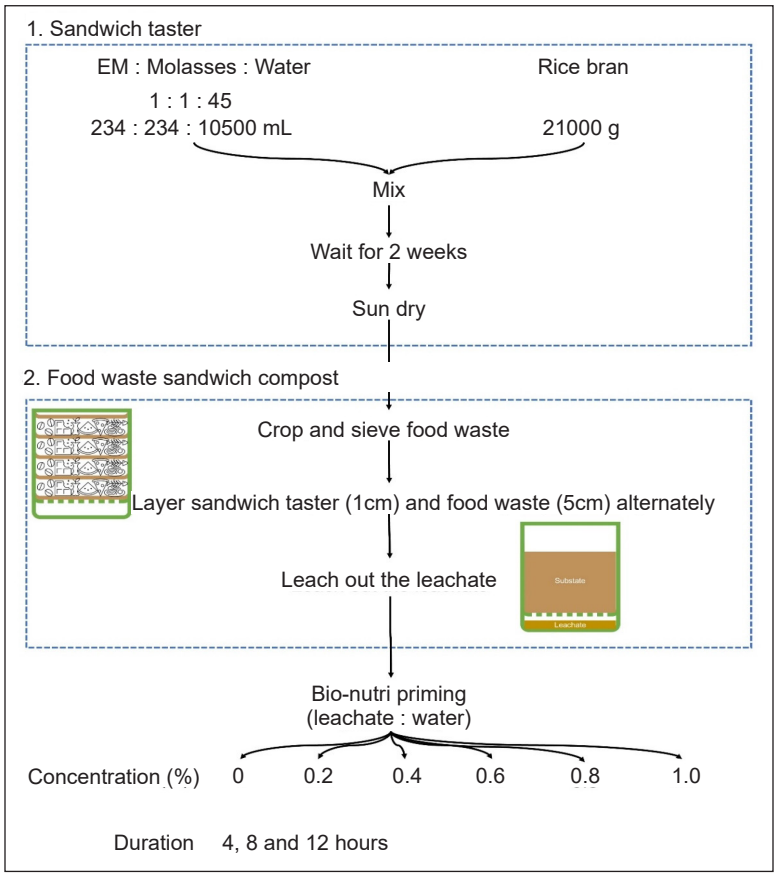


*Figure 1*. The procedure to prepare food waste sandwich compost leachate

### Statistical Analysis

Data were subjected to a two-way analysis of variance (ANOVA) using R software (version 4.1.2). If the *F* values were significant at the $p<0.10$ level, treatment means were compared and separated using the Fisher's least significant difference (LSD).

## RESULTS AND DISCUSSION

Seed priming was controlled by various factors such as priming agent concentration and duration (Waqas et al., 2019). Results indicated that the concentration of leachate and bio-nutri-priming duration showed a significant interaction on the standard error of germination rate ($S_{\overline{V}}$) and the corrected germination rate index ($S_{corrected}$) (Table 2).

A study has shown that micronutrient corn seed priming had significant interaction in the variables of germination percentage (GP), germination rate, the coefficient of the velocity of germination, days to germination, and mean germination time (Nciizah et al., 2020). However, in this study, there is no significant interaction between the different treatments on germination percentage (GP), germination rate as the reciprocal of the

median time ($V_{50}$), germination index (GI), and peak value (PV) (Table 2). Therefore, to obtain a better seedling vigour index, a 0.6% leachate concentration with a 12-hour bio-nutri-priming duration is recommended (Table 2).

Some studies state that nutrient toxicity might occur in seed coats with longer priming durations and high leachate concentration levels and is deleterious to the seed germination performance. For instance, corn seed metabolism changes with toxicity, thus, decreasing the utilisation of seed food reserves (Nciizah et al., 2020). Germination percentage was significantly reduced at a high concentration of micronutrient priming (0.5%) for a long duration (24 hours) (Nciizah et al., 2020). A 0.08% ginger rhizome aqueous extractant significantly decreased the germination percentage of chive (*Allium schoenoprasum* L.) (Han et al., 2008). Also, a 100 mM Zn micronutrient priming significantly reduced the seed germination percentage (Ajouri et al., 2004).

Micronutrient priming such as zinc, boron, and manganese significantly shortened the mean germination time in corn (Rasool et al., 2019). In addition, priming improved seed germination compounds production (Varier et al., 2010). For instance,

Table 2
*Germination performance based on bio-nutri-priming concentrations and duration*

| | GP (%) | $S_{\overline{V}}$ ($day^{-1}$) | $V_{50}$ ($day^{-1}$) | $S_{corrected}$ ($day^{-1}$) | GI | PV (% $day^{-1}$) | Seedling vigor index |
|---|---|---|---|---|---|---|---|
| **Concentrations (%)** | | | | | | | |
| 0.0 | 24.97b | 0.055a | 0.53a | 0.44a | 1.09b | 8.63b | 43.43b |
| 0.2 | 28.03ab | 0.066a | 0.58a | 0.45a | 1.26ab | 9.23ab | 45.96b |
| 0.4 | 28.75ab | 0.055a | 0.62a | 0.49a | 1.28ab | 0.98ab | 47.06b |
| 0.6 | 34.86a | 0.058a | 0.57a | 0.44a | 1.50a | 11.90a | 70.34a |
| 0.8 | 28.32ab | 0.065a | 0.57a | 0.44a | 1.16b | 9.48ab | 48.62ab |
| 1.0 | 31.77ab | 0.055a | 0.60a | 0.47a | 1.39ab | 11.12ab | 58.07ab |
| **Duration (hours)** | | | | | | | |
| 4 | 28.99a | 0.059ab | 0.54b | 0.43b | 1.23a | 9.28b | 47.63b |
| 8 | 27.57a | 0.054b | 0.58ab | 0.46ab | 1.26a | 9.72ab | 44.86b |
| 12 | 31.78a | 0.068a | 0.62a | 0.47a | 1.35a | 11.67a | 64.25a |
| **Significant level** | | | | | | | |
| Concentration (c) | ns | ns | ns | ns | ns | ns | ns |
| Duration (d) | ns | . | * | ns | ns | * | * |
| c×d | ns | ** | ns | * | ns | ns | . |
| Mean | 29.45 | 0.060 | 0.58 | 0.45 | 1.28 | 10.22 | 52.25 |
| Coefficient of variation (CV) | 31.29 | 31.81 | 18.72 | 14.62 | 28.23 | 33.57 | 50.87 |

*Note*. GP = Germination percentage; $S_{\overline{V}}$ = Standard error of germination rate; $V_{50}$ = Germination rate as the reciprocal of the median time; $S_{corrected}$ = Corrected germination rate index; GI = Germination index; PV = Peak value. Means with the same letter were not significantly different between treatments ($p$>0.05) using LSD. *** $p$<0.00; ** $p$<0.001; * $p$<0.01;. $p$<0.10; ns no significant $p$<1.00

DNA, RNA, and protein may be triggered to produce during the biopriming of corn (Afzal et al., 2008; Nciizah et al., 2020). Seed priming also enhanced seed protease and α-amylase activity for carbohydrates metabolism and eventually improved assimilation and translocation (Jafar et al., 2012).

Different bio-nutri-priming durations showed a significant positive effect on the germination rate as the reciprocal of the median time ($V_{50}$), peak value (PV), and seedling vigour index (Table 2). Bio-nutri-priming durations controlled the $V_{50}$, PV, and seed vigour index (Table 2). The longer the seed bio-nutri-priming duration, the better $V_{50}$, PV, and seed vigour index (Table 2). A long priming duration was key for enhanced germination despite studies showing that a prolonged nutrient priming duration may result in toxicity during seed germination (Nciizah et al., 2020). Hence, this study has demonstrated that seed bio-nutri-priming for 12 hours significantly improved the Chinese chive seed germination performance. Furthermore, because of long bio-nutri-priming, toxicity was not observed during the Chinese chive seed germination as the germination parameters significantly improved during the 12-hour bio-nutri-priming.

The 12-hour bio-nutri-priming duration had enhanced the germination rate of $V_{50}$, PV, and seedling vigour index, likely due to the dormant seed needing time to undergo the priming mechanism from imbibition, lag/activation, and germination phase. The 4- and 8-hour bio-priming duration may cut off the phases and be directed to the germination phase (Pawar & Laware, 2018). In the second lag phase, low water intake resulted from slight biomass improvement (Pawar & Laware, 2018). For instance, cabbage showed a high germination rate under 200 mmol $L^{-1}$ urea priming agent (Yan, 2015). Twelve (12) hours of Zn and Mn priming significantly improved the germination rate of marigold up to 93 % (Mirshekari et al., 2012).

The priming duration may vary between 8 hours to 14 days depending on different plant species, osmotic solution, osmotic potential, and temperature (Finch-Savage et al., 1991; Waqas et al., 2019). Nonetheless, an extended priming duration reduced soybean yield; hence, the suitable priming duration was 6 hours for germination performance and yield (Arif et al., 2008).

## CONCLUSION

Twelve hours of bio-nutri-priming duration with 0.6 % leachate is recommended for improved germination parameters in Chinese chives. However, this study was only limited to 10 days of seed germination. Thus, future studies can extend to the several harvest cycles to explore the significance between priming duration and leachate concentrations and better understand Chinese chives' nutrient utilisation capacity and accumulation. Furthermore, biotic stress could be applied to the bio nutrient of the sandwich compost leachate primed plant to understand the post-priming memory to later growth.

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## REFERENCES

Afzal, I., Rauf, S., Basra, S. M. A., & Murtaza, G. (2008). Halopriming improves vigor, metabolism of reserves and ionic contents in wheat seedlings under salt stress. *Plant, Soil and Environment*, *54*(9), 382–388. https://doi.org/10.17221/408-pse

Ajouri, A., Asgedom, H., & Becker, M. (2004). Seed priming enhances germination and seedling growth of barley under conditions of P and Zn deficiency. *Journal of Plant Nutrition and Soil Science*, *167*(5), 630–636. https://doi.org/10.1002/jpln.200420425

Aravind, J., Vimala Devi, S., Radhamani, J., Jacob, S. R., & Kalyani, S. (2019). *Germinationmetrics: Seed germination indices and curve fitting*. https://aravind-j.github.io/germinationmetrics/articles/Introduction.html

Arif, M., Jan, M. T., Marwat, K. B., & Khan, M. A. (2008). Seed priming improves emergence and yield of soybean. *Pakistan Journal of Botany*, *40*(3), 1169–1177.

Bisen, K., Keswani, C., Mishra, S., Saxena, A., Rakshit, A., & Singh, H. B. (2015). Unrealized potential of seed biopriming for versatile agriculture. In A. Rakshit, H. B. Singh, & A. Sen (Eds.), *Nutrient use efficiency: From basics to advances* (pp. 193–206). Springer. https://doi.org/10.1007/978-81-322-2169-2_13

Christel, D. M. (2017). *The use of bokashi as a soil fertility amendment in organic spinach cultivation*. https://scholarworks.uvm.edu/graddis/678

Davis, J. G., & Quick, J. S. (1998). Nutrient management, cultivar development and selection strategies to optimize water use efficiency. *Journal of Crop Production*, *1*(2), 221–240. https://doi.org/10.1300/J144v01n02_09

Finch-Savage, W., Gray, D., & Dickson, G. (1991). The combined effects of osmotic priming with plant growth regulator and fungicide soaks on the seed quality of five bedding plant species. *Seed Science and Technology*, *19*(2), 495–503.

Han, C. M., Pan, K. W., Wu, N., Wang, J. C., & Li, W. (2008). Allelopathic effect of ginger on seed germination and seedling growth of soybean and chive. *Scientia Horticulturae*, *116*(3), 330–336. https://doi.org/10.1016/j.scienta.2008.01.005

Harris, D., Raghuwanshi, B. S., Gangwar, J. S., Singh, S. C., Joshi, K. D., Rashid, A., & Hollington, P. A. (2001). Participatory evaluation by farmers of on-farm seed priming in wheat in India, Nepal and Pakistan. *Experimental Agriculture*, *37*(3), 403–415. https://doi.org/10.1017/S0014479701003106

Higa, T. (2001). *Effective microorganisms in the context of Kyusei Nature Farming – A technology for the future*. https://www.infrc.or.jp/knf/PDF%20KNF%20Conf%20Data/C6-KA-213.pdf

Higa, T., & Parr, J. F. (1994). *Beneficial and effective for a sustainable agriculture*. https://www.the-compost-gardener.com/support-files/em-1-higa-paper.pdf

Imran, M., Boelt, B., & Mühling, K. H. (2018). Zinc seed priming improves salt resistance in maize. *Journal of Agronomy and Crop Science*, *204*(4), 390–399. https://doi.org/10.1111/JAC.12272

Imran, M., Mahmood, A., Römheld, V., & Neumann, G. (2013). Nutrient seed priming improves seedling development of maize exposed to low root zone temperatures during early growth. *European Journal of Agronomy*, *49*, 141–148. https://doi.org/10.1016/j.eja.2013.04.001

International Seed Testing Association. (1995). *Understanding seed vigour*. ISTA. https://

www.seedtest.org/upload/prj/product/UnderstandingSeedVigourPamphlet.pdf

Jafar, M. Z., Farooq, M., Cheema, M. A., Afzal, I., Basra, S. M. A., Wahid, M. A., Aziz, T., & Shahid, M. (2012). Improving the performance of wheat by seed priming under saline conditions. *Journal of Agronomy and Crop Science*, *198*(1), 38–45. https://doi.org/10.1111/j.1439-037X.2011.00485.x

Kamenetsky, R., & Rabinowitch, H. D. (2017). Physiology of domesticated alliums: Onions, garlic, leek, and minor crops. In *Encyclopedia of applied plant sciences* (2nd ed., Vol. 3, pp. 255–261). Elsevier. https://doi.org/10.1016/B978-0-12-394807-6.00064-2

Liela, Y., Majid, A., & Fardin, K. (2010). Effect of seed priming duration and temperatureon seed germination behavior of bread wheat (*Triticum aestivum* L.). *Journal of Agricultural and Biological Science*, *5*(1), 1–6.

Makhaye, G., Aremu, A. O., Gerrano, A. S., Tesfay, S., Du Plooy, C. P., & Amoo, S. O. (2021). Biopriming with Seaweed extract and microbial-based commercial biostimulants influences seed germination of five *Abelmoschus esculentus* genotypes. *Plants*, *10*(7), 1327. https://doi.org/10.3390/plants10071327

Mirshekari, B., Baser, S., Allahyari, S., & Hamedanlu, N. (2012). On-farm seed priming with Zn + Mn is an effective way to improve germination and yield of marigold. *African Journal of Microbiology Research*, *6*(28), 5796-5800. https://doi.org/10.5897/ajmr12.256

Moeinzadeh, A., Sharif-Zadeh, F., Ahmadzadeh, M., & Tajabadi, F. H. (2010). Biopriming of sunflower (*Helianthus annuus* L.) seed with *Pseudomonas fluorescens* for improvement of seed invigoration and seedling growth. *Australian Journal of Crop Science*, *4*(7), 564–570.

Nciizah, A. D., Rapetsoa, M. C., Wakindiki, I. I., & Zerizghy, M. G. (2020). Micronutrient seed priming improves maize (*Zea mays*) early seedling growth in a micronutrient deficient soil. *Heliyon*, *6*(8), e04766. https://doi.org/10.1016/j.heliyon.2020.e04766

Olle, M. (2020). Short communication: The improvement of the growth of tomato transplants by bokashi tea. *Agraarteadus*, *31*(1), 70–73. https://doi.org/10.15159/jas.20.10

Paparella, S., Araújo, S. S., Rossi, G., Wijayasinghe, M., Carbonera, D., & Balestrazzi, A. (2015). Seed priming: State of the art and new perspectives. *Plant Cell Reports*, *34*(8), 1281–1293. https://doi.org/10.1007/s00299-015-1784-y

Pawar, V. A., & Laware, S. L. (2018). Seed priming a critical review. *International Journal of Scientific Research in Biological Sciences*, *5*(5), 94–101. https://doi.org/10.26438/ijsrbs/v5i5.94101

Phooi, C. L., Azman, E. A., & Ismail, R. (2021). *Bokashi leachate as a biopriming on* Basella rubra *L. seed germination and root development*. https://doi.org/10.21203/RS.3.RS-855828/V1

Rasool, T., Ahmad, R., & Farooq, M. (2019). Seed priming with micronutrients for improving the quality and yield of hybrid maize. *Gesunde Pflanzen*, *71*(1), 37–44. https://doi.org/10.1007/s10343-018-00440-8

Shah, H., Jalwat, T., Arif, M., & Miraj, G. (2012). Seed priming improves early seedling growth and nutrient uptake in mungbean. *Journal of Plant Nutrition*, *35*(6), 805–816. https://doi.org/10.1080/01904167.2012.663436

Sun, S., Zhang, W., & Liu, T. (2010). 赤霉素对韭菜种子萌发和早期幼苗生长的影响 [Effects of gibberellin on the germination and early seedling growth of *Allium tuberosum* Rottl.ex Spreng.]. 种子世界, *8*, 35–36.

Tang, X., Olatunji, O. J., Zhou, Y., & Hou, X. (2017). *Allium tuberosum*: Antidiabetic and hepatoprotective activities. *Food Research International*, *102*, 681–689. https://doi.org/10.1016/j.foodres.2017.08.034

Toribio, A. J., Jurado, M. M., Suárez-Estrella, F., López, M. J., López-González, J. A., & Moreno, J. (2021). Seed biopriming with cyanobacterial extracts as an eco-friendly strategy to control damping off caused by *Pythium ultimum* in seedbeds. *Microbiological Research*, *248*, 126766. https://doi.org/10.1016/j.micres.2021.126766

Varier, A., Vari, A. K., & Dadlani, M. (2010). The subcellular basis of seed priming. *Current Science*, *99*(4), 450–456. https://www.jstor.org/stable/24109568

Wang, Y., Raghavan, S., & Ho, C.-T. (2008). Process flavors of *Allium* vegetables. In *Fruit and vegetable flavour: Recent advances and future prospects* (pp. 200 –226). Woodhead Publishing. https://doi.org/10.1533/9781845694296.3.200

Waqas, M., Korres, N. E., Khan, M. D., Nizami, A.-S., Deeba, F., Ali, I., & Hussain, H. (2019). Advances in the concept and methods of seed priming. In M. Hasanuzzaman & V. Fotopoulos (Eds.), *Priming and pretreatment of seeds and seedlings* (pp. 11–41). Springer. https://doi.org/10.1007/978-981-13-8625-1_2

Yan, M. (2015). Seed priming stimulate germination and early seedling growth of Chinese cabbage under drought stress. *South African Journal of Botany*, *99*, 88–92. https://doi.org/10.1016/j.sajb.2015.03.195

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# Using *Streptomyces* spp. as Plant Growth-Promoting Inoculants for Growth of Napier Grass under Low Water System

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## ABSTRACT

Napier grass can be used as feed for livestock and possibly for bioenergy production. However, the stimulation of the growth of Napier grass by plant growth-promoting bacteria (PGPB) has been rarely found. Thus, this study was performed to investigate the ability of *Streptomyces* spp. PB5, SRF1, St8, STRM104, and STRM302 to support the growth of Napier grass (*Pennisetum purpureum* × *Pennisetum americanum* cultivar Pak Chong 1) under a low water system. Among the five bacterial isolates, *Streptomyces* sp. St8 was the most suitable bacterial inoculant to stimulate the growth of plants grown under a low water system. Napier grass grew under a low water system and inoculated with *Streptomyces* sp. St8 had the highest shoot and root weight compared to the other inoculated isolates. The shoot and root fresh weights of plants grown under a low water system were 21.3 ± 1.53 g and 4.29 ± 0.77 g when inoculated with *Streptomyces* sp. St8. Moreover, *Streptomyces* sp. St8 also stimulated the growth of plants grown under a normal water system: the highest shoot length (61.3 ± 5.67 cm), shoot fresh weight (26.9 ± 4.07 g), and root fresh weight (4.84 ± 0.54 g) were found in plants inoculated with this bacterial isolate. Furthermore, the plant's root-to-shoot ratios grown under a low water system were inoculated with each isolate of *Streptomyces* sp. (PB5, SRF1, St8, STRM104, and STRM302) were lower than for plants grown in the control pots. It means that bacterial inoculation under a low water

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system could protect the efficiency of roots from producing shoot biomass in the plants. Based on the results found in this study, *Streptomyces* sp. St8, a microbial inoculant, can be used with Napier grass cropping to produce feed for livestock or bioenergy production.

*Keywords*: Low water, Napier grass, plant growth-promoting bacteria, *Streptomyces*

## INTRODUCTION

Napier grass is a fast-growing perennial grass usually found in humid soils in areas with over 1,000 mm of rainfall per year. Napier grass is a stress-tolerant forage crop, including plant disease and short drought stresses, and it can grow under low fertility (Negawo et al., 2017; Odiyi & Oludare, 2015). In Thailand, it is mainly used to feed livestock, and it is expected to be used for other purposes, including as a substrate for bioenergy production and biomass for electricity generation (Nantasaksiri et al., 2021; Osman et al., 2020; Waramit & Chaugool, 2014). Some genotypes of Napier grass can generate large biomass and accumulate nitrogen derived from biological nitrogen fixation when grown under low levels of nitrogen in the soil (Videira et al., 2012). Information about the possibility of using Napier grass as a resource for bioenergy production in Thailand is required in numerous areas for plantations. Moreover, biomass production from Napier grass for bioenergy production cannot compete with food or forage crop production for arable land. Thus, bioenergy crops should be grown on non-fertile soils, which are not appropriate for other economic crops (Mei et al., 2021). Using plant growth-promoting bacteria (PGPB) is one way to improve plant growth and yield under unfavorable conditions. The application of PGPB to stimulate the growth of Napier grass has been rarely found, even though several PGPB have been isolated from Napier grass, including diazotrophic nitrogen-fixing bacteria belonging to the genera *Azospirillum* and *Gluconacetobacter* (Videira et al., 2012). PGPB from the genera *Bacillus*, *Enterobacter*, and *Sphingomonas* can solubilize insoluble phosphate, fix nitrogen, produce indole-3-acetic acid, ammonia, and siderophores have also been isolated from Napier grass, which could enhance salt tolerance in hybrid *Pennisetum* (Li et al., 2016).

The objective of this study was to investigate the ability of five isolates of *Streptomyces* spp. (PB5, SRF1, St8, STRM104, and STRM302) to stimulate the growth of Napier grass under low water conditions. The reason for using *Streptomyces* spp. as a model PGPB in this study was that many species had been shown to alleviate undesirable effects from drought stress on the plants in Gramineae. For example, *Streptomyces coelicolor* DE7, *Streptomyces olivaceus* DE10, and *Streptomyces geysiriensis* DE27 have been previously isolated from arid and drought-affected areas, and they could promote the growth of wheat cultivar WR-544 when grown in water-stress soil using the combined effects from phytohormone

production and water stress tolerance ability (Yandigeri et al., 2012). In addition, *Streptomyces pseudovenezuelae* MG547870 could produce indole-3-acetic acid and ACC deaminase, and it could increase the growth and alleviate severe effects from drought on maize (Chukwuneme et al., 2020). Moreover, *Streptomyces albidoflavus* OsiLf-2 increased the osmotic modification ability of rice grown under drought and salt stresses by increasing proline and sugar content in the plant (Niu et al., 2022). Even though the five isolates of *Streptomyces* spp. used in this study have never been tested to promote the growth of Napier grass previously, all isolates have plant growth-promoting activities. For example, *Streptomyces* sp. St8, STRM104, and STRM302 can solubilize phosphate and produce indole-3-acetic acid (Somtrakoon et al., 2019a, 2021). *Streptomyces* sp. SRF1 has only indole-3-acetic acid production activity (Somtrakoon et al., 2019a) during *Streptomyces* sp. PB5 has never been tested for plant growth-promoting activity, but it was tested in this study. Moreover, these five bacterial isolates have not been isolated from Napier grass. However, if they can stimulate the growth of Napier grass under low water, a biofertilizer from bacteria in this genus may be developed for Napier grass planting in the future.

## MATERIALS AND METHODS

### Plant Growth-Promoting Activity

Five isolates of *Streptomyces* spp., PB5, SRF1, St8, STRM104, and STRM302, were kindly provided by the Microbiology and Applied Microbiology Research Unit, Faculty of Science, Mahasarakham University. Each *Streptomyces* sp. isolate was isolated from different agricultural areas in Thailand. *Streptomyces* sp. SRF1 (Sangdee et al., 2016) and PB5 were isolated from paddy field and integrated agricultural area in Lopburi and Buriram Provinces, respectively. *Streptomyces* sp. St8 was isolated from soil planted with a mango tree in Kalasin Province. *Streptomyces* sp. STRM104 and STRM302 were isolated from soil planted with tomatoes in Maha Sarakham Province. Each isolate of *Streptomyces* sp. was sub-cultured in half-strength potato dextrose agar (PDA) [potato dextrose broth powder (Himedia™, India) 12 g, agar powder (Difto, USA) 20 g, distilled water 1,000 ml, and the pH was adjusted to 7.0]. Then, the plant growth-promoting activities of *Streptomyces* sp. PB5 to solubilize phosphate, produce indole-3-acetic acid and ammonia were tested using the methods described in Ahmad et al. (2008), while the exopolysaccharide producing activity was tested using the methods described in Lakshminarayanan et al. (2015). Only the exopolysaccharide and ammonia-producing activities of *Streptomyces* sp. SRF1, St8, STRM104 and STRM302 were tested using the methods described in Lakshminarayanan et al. (2015) and Ahmad et al. (2018).

### Preparation of Bacterial Culture

To prepare the bacterial inoculum used in the pot experiment, *Streptomyces* spp. PB5, SRF1, St8, STRM104, and STRM302

were grown in half-strength PDA, pH 7.0, and incubated at 37 ºC for 14 days. Approximately 2–3 ml of 0.85% sodium chloride (NaCl) + 0.1% Tween 80 solution were poured onto the agar surface, and the cells and spores of each isolate of *Streptomyces* sp. were scraped with a loop and re-suspended in 0.85% NaCl + 0.1% Tween 80 solution (adapted from Somtrakoon et al., 2019b). A suspension of cells and spores was transferred into the culture tube, and the optical density was adjusted to be 0.5 at an optical wavelength at 600 nm. The initial cell number of each bacterial isolate of *Streptomyces* sp. from the culture suspension was serial diluted and counted on half-strength PDA by the drop plate method before use as an inoculum. The initial cell numbers of each isolate of *Streptomyces* sp. used to prepare the bacteria suspension in the pot experiment for the first and the second inoculations were recorded (Table 1).

**Preparation of Soil**

The soil used in this study was collected from wasteland in Khamriang Sub-district, Khantharawichai District, Maha Sarakham Province, Thailand. The soil was air-dried for two weeks before use. After serial dilution, the total heterotrophic bacteria in the soil used in this study were counted on nutrient agar using the spread plate method. At the beginning of the experiment, the number of total heterotrophic bacteria was $5.3\times10^4$ CFU/g dry soil. Then, these soils were sub-divided into the experimental pots, with each experimental pot containing 4 kg of dry soil. There were 120 pots for the experiment.

**Experimental Design**

The ability of each isolate of *Streptomyces* sp. to stimulate the growth of Napier grass was determined in a 2 x 6 factorial, completely randomized design with ten replicates. Two factors were two levels of the water system (normal water and low water irrigation) × six levels of bacterial inoculation (non-inoculation and inoculation with PB5, SRF1, St8, STRM104, and STRM302). The details of each treatment are given in Table 2.

**Pot Experiment**

Stems of Napier grass cultivar 'Pak Chong 1' were cut into 13-14 cm pieces, with each piece having only one node and then soaked

Table 1
*Initial cell numbers of* Streptomyces *spp. used in pot experiments*

| Bacterial isolates | 1st inoculation (CFU/ml) (14 days after transplantation) | 2nd inoculation (CFU/ml) (31 days after transplantation) |
|---|---|---|
| *Streptomyces* sp. PB5 | $8.7 \times 10^{10}$ | $8.7 \times 10^{10}$ |
| *Streptomyces* sp. SRF1 | $2.5 \times 10^{10}$ | $1.9 \times 10^{10}$ |
| *Streptomyces* sp. St8 | $3.5 \times 10^{8}$ | $3.3 \times 10^{8}$ |
| *Streptomyces* sp. STRM104 | $1.0 \times 10^{10}$ | $9.3 \times 10^{9}$ |
| *Streptomyces* sp. STRM302 | $4.3 \times 10^{9}$ | $4.3 \times 10^{9}$ |

Table 2
*Details of each treatment*

| Treatment | Water system | *Streptomyces* isolate |
|---|---|---|
| 1 | Normal water | Non-inoculation |
| 2 | Normal water | PB5 |
| 3 | Normal water | SRF1 |
| 4 | Normal water | St8 |
| 5 | Normal water | STRM104 |
| 6 | Normal water | STRM302 |
| 7 | Low water | Non-inoculation |
| 8 | Low water | PB5 |
| 9 | Low water | SRF1 |
| 10 | Low water | St8 |
| 11 | Low water | STRM104 |
| 12 | Low water | STRM302 |

in water for 72 hours. One cutting of Napier grass was planted in each experimental pot until the young plant was 14 days old. At this age, 2 ml of each bacterial inoculum (Table 1) was mixed with 250 ml of water and poured into the experimental pot. Pots that did not receive the bacterial inoculum had distilled water added as a non-inoculated control. The water system was set into two patterns; 250 ml of water was added to the experimental pot once every three days for the normal water system and once every six days for the low water system. The second bacterial inoculation was performed one month after planting. Again 2 ml of each bacterial inoculum (Table 1) was mixed with 250 ml of water and poured into the planted soil. Napier plants were grown until they were 49 days old when the experiment was terminated. Then, the physical and chemical characteristics of the soil in a low water system at the end of the experiment were analyzed at the Soil-Fertilizer-Environment Academic Development Project, Department of Soil Science, Kasetsart University, Bangkok, Thailand.

### Plant Growth Measurement

Plant growth parameters were determined at the end of the experiment, including shoot length, root length, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, and the number of leaves. Total chlorophyll, chlorophyll *a*, and chlorophyll *b* contents in leaves of Napier plants were determined according to the methods described in Huang et al. (2004). The relative water content (RWC) in the leaves of the Napier plants was analyzed according to the methods described in Sade et al. (2015). The specific root length was calculated from the root length/root dry weight (Calvelo Pereira et al., 2010). The root to shoot ratio was calculated from the root dry weight/shoot dry weight (Xu et al., 2018).

### Statistical Analysis

A two-way analysis of variance (ANOVA) and least square difference (LSD) tests were used for variance analysis and pairwise comparison for plant growth. Microsoft Excel was used for statistical analysis.

## RESULTS AND DISCUSSION

### Relative Water Content and Chlorophyll Content in Leaves

The growth levels of Napier grass planted under normal and low water systems in this study were similar. This study did not change

Napier grass's growth under the low water system. The RWC confirmed it in Napier grass leaves that were not significantly different between normal and low water systems for the same bacterial isolate (Table 3). However, RWC in leaves differed between some inoculations within the same water system, for example, *Streptomyces* sp. St8 and STRM302 under the normal water system, and non-inoculation and *Streptomyces* sp. STRM104 under the low water system. Normally, the RWC in leaves of plants decreases when encountering drought conditions (Machado & Paulsen, 2001). It may be due to Napier grass being tolerant to short droughts. It has been reported that Napier grass could survive under non-irrigated conditions and could generate higher biomass during the dry season than in the rainy season (Haegele et al., 2017).

Under the normal water system, inoculation of Napier grass with *Streptomyces* sp. isolates PB5, SRF1, St8, and STRM104

Table 3
*Chlorophyll content and relative water content of Napier grass leave grown under normal system and low water condition for 49 days [mean ± standard error (SE)]*

| **Treatment** | Chlorophyll *a* (mg/ml) | Chlorophyll *b* (mg/ml) | Total chlorophyll (mg/ml) | RWC (%) |
|---|---|---|---|---|
| Normal water system | | | | |
| Control | 5.09 ± 1.02cA | 6.81 ± 0.39cB | 11.90 ± 1.42dB | 78.2 ± 21.6abA |
| PB5 | 12.32 ± 1.13abA | 9.84 ± 0.63bA | 22.15 ± 0.50bA | 58.5 ± 8.5abA |
| SRF1 | 10.00 ± 0.29bA | 6.85 ± 0.07cA | 16.85 ± 0.32cA | 51.2 ± 13.6abA |
| St8 | 16.14 ± 2.08aA | 16.35 ± 1.12aA | 32.48 ± 1.14aA | 85.9 ± 9.8aA |
| STRM104 | 11.80 ± 1.29bA | 10.19 ± 0.38bB | 21.98 ± 1.06bB | 49.7 ± 19.2abA |
| STRM302 | 4.09 ± 1.12cA | 6.20 ± 0.04cA | 10.29 ± 1.09dA | 20.7 ± 13.1bA |
| Low water system | | | | |
| Control | 10.85 ± 1.57abA | 16.99 ± 0.97aA | 27.83 ± 2.36aA | 96.9 ± 11.5aA |
| PB5 | 14.87 ± 0.29aA | 8.17 ± 0.21cA | 23.03 ± 0.50bA | 57.5 ± 28.8abA |
| SRF1 | 8.00 ± 0.53bA | 6.81 ± 0.35cdA | 14.81 ± 0.24cA | 64.8 ± 14.1abA |
| St8 | 14.86 ± 3.23aA | 13.19 ± 2.33bB | 28.04 ± 1.43aB | 76.5 ± 9.8abA |
| STRM104 | 14.52 ± 0.74aA | 14.77 ± 0.20abA | 29.29 ± 0.70aA | 48.2 ± 22.5bA |
| STRM302 | 4.62 ± 0.48bA | 5.04 ± 0.15dA | 9.66 ± 0.55dA | 59.1 ± 6.4abA |
| Water system | ns | ** | ** | ns |
| Bacteria | ** | ** | ** | * |
| Water system x bacteria | ns | ** | ** | ns |

*Note*. Different lower-case letters show significant differences between inoculations of bacterial isolates under the same water system ($P<0.05$), and different capital letters show significant differences between normal system and low water system with the same bacterial isolate inoculations ($P<0.05$). Abbreviations: ns, *, ** denote non-significance ($P>0.05$), statistical significance ($P<0.05$), and high statistical significance ($P<0.01$) for each factor, respectively

increased the total chlorophyll content in the leaves of the plant when compared to the control pots (Table 5). The highest total chlorophyll content in the plant's leaves was observed in soil inoculated with St8. Under the low water system, inoculation of St8 and STRM104 could maintain the chlorophyll content in the leaves of Napier grass because the total chlorophyll content in the leaves of plant inoculation with *Streptomyces* sp. isolates St8 (28.04 ±1.43 mg/ml) and STRM104 (29.29 ± 0.70 mg/ml) were not significantly different from the control pots (27.83 ± 2.36 mg/ml). However, the total chlorophyll content in the leaves of plants inoculated with *Streptomyces* sp. SRF1, STRM302, and PB5 were lower than the total chlorophyll content in the plant's leaves in the control pots (Table 3). Normally, drought stress decreases the chlorophyll content in plants (Chandra et al., 2018), but a decrease in the chlorophyll content in the low water system was only found in the leaves of plants inoculated with *Streptomyces* sp. St8. On the other hand, the chlorophyll content in the leaves of plants inoculated with *Streptomyces* sp. STRM104 and non-inoculated plants were increased in the low water system.

**Shoot and Root Growth of Napier Grass**

The leaf numbers of Napier grass grown under the normal water system were similar between the control pots and pots inoculated with each bacterial isolate. However, decreased leaf numbers were found in plants grown in the control pots under the low water system (Table 4). This phenomenon is prominently found in plants grown under drought stress because decreasing the leaf number is one of the adaptation mechanisms in plants. In general, the plant responds to drought via many adaptations in the leaves to limit water loss, such as thickening the palisade parenchyma in the leaf, decreasing the leaf area, stomatal size, and leaf number (Deblonde & Ledent, 2001; Zhang et al., 2018). Surprisingly, using *Streptomyces* sp. PB5, St8, and STRM104 could increase the leaf number of plants grown under the low water system to be comparable to plants grown under the normal water system. It corresponds to the results of shoot growth because increasing shoot growth was also observed in the experimental pot inoculation with *Streptomyces* sp. PB5, St8, STRM104, and STRM302 under normal and low water systems (Table 4). Application of *Streptomyces* sp. St8 under both normal and low water systems tended to give the highest shoot fresh weight (26.9 ± 4.07 g and 21.3 ± 1.53 g) and shoot dry weight (3.60 ± 0.540 g and 2.84 ± 0.190 g) compared to the inoculation with the other bacterial isolates (Table 4 and Figure 1). Moreover, the highest root growth in fresh and dry weight was also observed in the experimental pots inoculated with *Streptomyces* sp. St8 under both normal and low water systems (Table 4). The root's fresh and dry weights were 4.29 ± 0.77 g and 0.62 ± 0.099 g when the soil was inoculated with *Streptomyces* sp. St8 under the low water system. However, *Streptomyces* sp. SRF1 was unsuitable as a microbial inoculant for Napier grass cultivation. This bacterial isolate stimulated the growth of

Table 4
*Shoot and root growth of Napier grass grown under normal water and low water systems for 49 days [mean ± standard error (SE)]*

| | Leaf number | Shoot length (cm) | Shoot fresh weight (g) | Shoot dry weight (g) | Root length (cm) | Root fresh weight (g) | Root dry weight (g) | Root to shoot ratio | Specific root length (m/g) |
|---|---|---|---|---|---|---|---|---|---|
| Normal water | | | | | | | | | |
| Control | 8.4 ± 0.40 | 41.0 ± 1.57bA | 11.5 ± 1.24cA | 1.25 ± 0.150bA | 48.6 ± 5.60aA | 2.49 ± 0.42bA | 0.23 ± 0.042cA | 0.19 | 2.09 |
| PB5 | 8.5 ± 0.55 | 61.1 ± 6.39aA | 20.2 ± 2.19bA | 1.92 ± 0.328bA | 45.2 ± 4.51aA | 3.35 ± 0.60bA | 0.48 ± 0.087bA | 0.25 | 0.93 |
| SRF1 | 8.0 ± 0.24 | 53.0 ± 2.34abA | 15.7 ± 1.53bcA | 1.99 ± 0.252bA | 50.8 ± 3.70aA | 3.62 ± 0.70abA | 0.50 ± 0.100bA | 0.21 | 1.02 |
| St8 | 9.3 ± 0.42 | 61.3 ± 5.67aA | 26.9 ± 4.07aA | 3.60 ± 0.540aA | 43.5 ± 4.35aA | 4.84 ± 0.54aA | 0.76 ± 0.119aA | 0.24 | 0.57 |
| STRM104 | 9.3 ± 0.37 | 56.8 ± 5.62aA | 20.3 ± 2.09bA | 2.63 ± 0.428abA | 47.9 ± 3.64aA | 2.79 ± 0.26bA | 0.63 ± 0.150abA | 0.20 | 0.76 |
| STRM302 | 8.6 ± 0.50 | 57.3 ± 5.38aA | 19.6 ± 1.63bA | 2.59 ± 0.357abA | 50.6 ± 2.11aA | 3.80 ± 0.48abA | 0.53 ± 0.070abA | 0.25 | 0.95 |
| Low water | | | | | | | | | |
| Control | 6.9 ± 0.43 | 40.8 ± 3.57bcA | 9.7 ± 1.44cA | 1.17 ± 0.194bA | 39.3 ± 3.12cA | 2.67 ± 0.41bA | 0.13 ± 0.049bA | 0.27 | 1.24 |
| PB5 | 9.0 ± 0.30 | 58.4 ± 4.09abA | 16.0 ± 1.08bA | 2.24 ± 0.165aA | 45.2 ± 2.58bcA | 2.29 ± 0.26bA | 0.25 ± 0.036bA | 0.11 | 1.78 |
| SRF1 | 6.5 ± 0.58 | 36.8 ± 4.11cA | 8.9 ± 1.34cB | 1.17 ± 0.227bA | 57.8 ± 6.44aA | 2.00 ± 0.25bB | 0.25 ± 0.036bB | 0.22 | 2.27 |
| St8 | 8.9 ± 0.23 | 59.0 ± 4.22abA | 21.3 ± 1.53aB | 2.84 ± 0.190aA | 48.4 ± 1.71abA | 4.29 ± 0.77aA | 0.62 ± 0.099aA | 0.24 | 0.71 |
| STRM104 | 9.3 ± 0.17 | 66.5 ± 4.92aA | 18.2 ± 1.13abA | 2.23 ± 0.196aA | 36.6 ± 3.53cA | 2.45 ± 0.22bA | 0.46 ± 0.065abA | 0.21 | 0.79 |
| STRM302 | 7.3 ± 0.59 | 52.2 ± 3.58bA | 16.6 ± 1.36abA | 2.16 ± 0.254aA | 48.9 ± 2.90abA | 1.84 ± 0.31bB | 0.38 ± 0.056bA | 0.12 | 1.28 |
| Water | | ns | ** | ns | ns | * | * | | |
| Bacteria | | ** | ** | ** | * | ** | ** | | |
| Water x bacteria | | ns | ns | ns | ns | ns | ns | | |

*Note*. Different lower-case letters show significant differences between inoculations of bacterial isolates under the same water system ($P<0.05$), and different capital letters show significant differences between normal system and low water system with the same bacterial isolate inoculations ($P<0.05$). The data were not normally distributed for leaf number, and the statistical calculation was not performed. Abbreviations: ns, *, ** denote non-significance ($P>0.05$), statistical significance ($P<0.05$), and high statistical significance ($P<0.01$) of each factor, respectively

plants grown under both normal and low water systems to a lesser extent than the other isolates (Table 4 and Figure 1). It may be due to no phosphate solubilization activity detected in *Streptomyces* sp. SRF1 and only a slight level of indole-3-acetic acid were produced by this bacterial isolate (Somtrakoon et al., 2019a).

The stimulation of the growth of Napier grass in this study may be due to the plant growth-promoting activities of *Streptomyces*. Our previous work (Somtrakoon et al., 2019a, 2021), and recent tests on plant growth-promoting activity, revealed that *Streptomyces* sp. St8, STRM104, STRM302, and PB5 can produce indole-3-acetic acid, exopolysaccharide, ammonia, and solubilize phosphate (Table 5). These activities assist in promoting the growth of plants by several mechanisms. For example, IAA production supports plant growth by increasing root growth, which permits the plant to uptake more soil nutrients (Goswami et al., 2013). In addition, increasing the soil water holding capacity by bacterial exopolysaccharides promotes plant

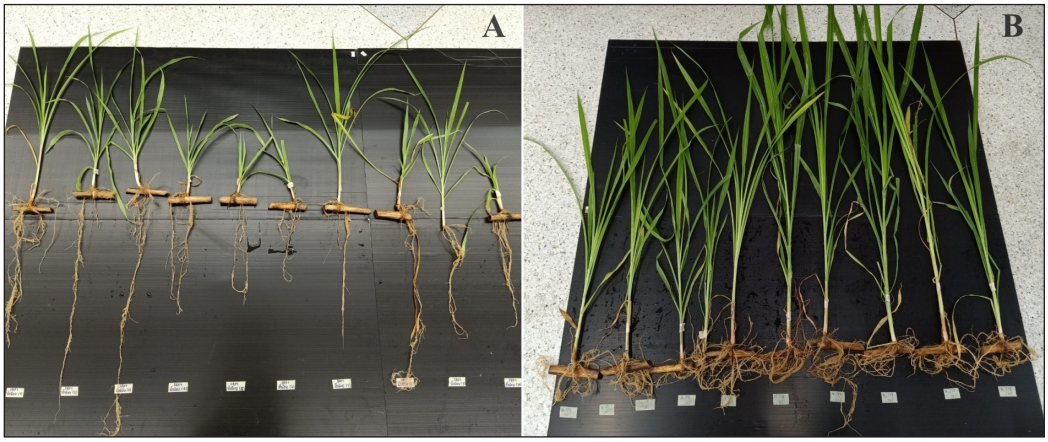


*Figure 1*. The 49-day-old Napier grass grown under a low water system when inoculated with *Streptomyces* sp. SRF1 (A) and St8 (B), respectively

Table 5
*Plant growth-promoting activity of* Streptomyces *sp. PB5, SRF1, St8, STRM104, and STRM302*

| Bacteria | IAA production | Phosphate solubilization | Exopolysaccharide production | Ammonia Production |
|---|---|---|---|---|
| PB5 | + | + | + | + |
| SRF1 | ND[A] | ND[A] | + | + |
| St8 | ND[A] | ND[A] | + | + |
| STRM104 | ND[B] | ND[B] | + | + |
| STRM302 | ND[B] | ND[B] | + | + |

*Note*. ND[A] mean not determined in this study. Plant growth-promoting activity was determined in Somtrakoon et al. (2019a); ND[B] mean not determined in this study. Plant growth-promoting activity was determined in Somtrakoon et al. (2021); Symbols + and - indicate positive and negative activities, respectively

growth via increasing the nutrient uptake and aiding the colonization of PGPB to the plant root zone (A. Kumar et al., 2020; Khan et al., 2017). Bacterial colonization of plant roots is a significant procedure for PGPB to survive, grow, and function in the soil (de Souza et al., 2015). In addition, increasing phosphorus mobilization by PGPB could promote phosphorus uptake by plants and support plants grown in soil (Pereira et al., 2020). The ammonia-producing ability of PGPB also provides a nitrogen source for plants (Goswami et al., 2013), and it can act to protect the plants from phytopathogens (Fahsi et al., 2021).

In general, indigenous bacteria have been proposed to be used as microbial inoculants because of their adaptation capacity to the environment after inoculation into the environment again (B. L. Kumar & Gopal, 2015). However, the results of this study confirmed that the *Streptomyces* sp., which has not previously been isolated from soil planted with Napier grass, could promote the growth of plants to an obvious extent compared to the control. *Streptomyces* sp. St8 was the most suitable microbial inoculant for Napier grass planting based on the root to shoot ratio. It is confirmed by a similar root to shoot ratio of plant inoculation with *Streptomyces* sp. St8, which was similar between the normal and low water system conditions. It means that growing under a low water system did not affect the integrity of the root of Napier grass. The root to shoot ratio of Napier grass inoculation with *Streptomyces* sp. STRM104 was also constant between the normal water and low water systems, but the ability to stimulate the growth of Napier grass by this bacterial isolate was poor. Meanwhile, the root to shoot ratio of the plants in the control pots was increased under the low water system. It means that the roots of Napier grass grown under a low water system were not healthy. Therefore, using *Streptomyces* sp. St8 is the best to protect the root integrity of the plant in this study. However, the nutrient elements in all soils planted with Napier grass and inoculated with each isolate of *Streptomyces* sp. were lower than those in soil planted with Napier grass only (Table 6). The soil organic matter, available phosphorus, exchangeable potassium, exchangeable calcium, exchangeable magnesium, and total nitrogen in planted soil inoculated with *Streptomyces* sp. PB5, SRF1, St8, STRM104, and STRM302 were not increased compared to the control pots (Table 6). Available phosphorus, exchangeable potassium, and exchangeable calcium in the control pots were higher than those inoculated with *Streptomyces* sp. PB5, SRF1, St8, STRM104, and STRM302.

## CONCLUSION

Inoculation with *Streptomyces* could increase Napier grass growth, and it is possible to use it as a biofertilizer for Napier grass planting. The different bacterial isolates had important factors that affect the Napier grass's growth and *Streptomyces* sp. St8 was the best isolate. The different systems in this study did not decrease the Napier grass's growth. For Napier grass inoculated with *Streptomyces* sp. St8, only

Table 6
*Physical and chemical characteristics of soil under low water condition after Napier grass planting for 49 days*

| Treatment | pH | Calcium carbonate requirement ($CaCO_3$/rai) | Organic matter (g/kg) | % sand | % silt | % clay | Soil texture | Available phosphorus (mg/kg) | Exchangeable potassium (mg/kg) | Exchangeable calcium (mg/kg) | Exchangeable magnesium (mg/kg) | Total nitrogen (g/kg) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 3.88 | 403 | 2.2 | 66 | 21 | 13 | Sandy loam | 6.1 | 72 | 813 | 39 | 0.26 |
| PB5 | 3.99 | 403 | 2.0 | 71 | 18 | 11 | Sandy loam | 4.6 | 30 | 354 | 30 | 0.26 |
| SRF1 | 4.01 | 403 | 2.8 | 70 | 18 | 12 | Sandy loam | 5.3 | 57 | 475 | 35 | 0.26 |
| St8 | 3.96 | 403 | 2.1 | 70 | 19 | 11 | Sandy loam | 4.6 | 26 | 587 | 33 | 0.17 |
| STRM104 | 3.98 | 269 | 2.1 | 70 | 19 | 11 | Sandy loam | 5.3 | 37 | 399 | 30 | 0.22 |
| STRM302 | 4.07 | 403 | 1.9 | 69 | 19 | 12 | Sandy loam | 4.2 | 29 | 378 | 33 | 0.22 |

the shoot fresh weight was decreased in the low system condition. Even though inoculation of soil with *Streptomyces* sp. did not increase the planted soil's fertility in this study, the nutrient accumulation in Napier grass inoculated with *Streptomyces* should be analyzed in further experiments.

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## REFERENCES

Ahmad, F., Ahmad, I., & Khan, M. S. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research*, *163*(2), 178-181. https://doi.org/10.1016/j.micres.2006.04.001

Calvelo Pereira, R., Monterroso, C., & Macías, F. (2010). Phytotoxicity of hexachlorocyclohexane: Effect on germination and early growth of different plant species. *Chemosphere*, *79*(3), 326–333. https://doi.org/10.1016/j.chemosphere.2010.01.035

Chandra, D., Srivastava, R., Glick, B. R., & Sharma, A. K. (2018). Drought-tolerant *Pseudomonas* spp. improve the growth performance of finger millet (*Eleusine coracana* (L.) Gaertn.) under non-stressed and drought-stressed conditions. *Pedosphere*, *28*(2), 227-240. https://doi.org/10.1016/S1002-0160(18)60013-X

Chukwuneme, C. F., Babalola, O. O., Kutu, F. R., & Ojuederie, O. B. (2020). Characterization of actinomycetes isolates for plant growth promoting traits and their effects on drought tolerance in maize. *Journal of Plant Interaction*, *15*(1), 93-105. https://doi.org/10.1080/17429145.2020.1752833

de Souza, R., Ambrosini, A., & Passaglia. L. M. P. (2015). Plant growth-promoting bacteria as inoculants in agricultural soils. *Genetics and Molecular Biology*, *38*(4), 401-419. https://doi.org/10.1590/S1415-475738420150053

Deblonde, P. M. K., & Ledent, J. F. (2001). Effects of moderate drought conditions on green leaf number, stem height, leaf length and tuber yield of potato cultivars. *European Journal of Agronomy*, *14*(1), 31-41. https://doi.org/10.1016/S1161-0301(00)00081-2

Fahsi, N., Mahdi, I., Mesfioui, A., Biskri, L., & Allaoui, A. (2021). Phosphate solubilizing rhizobacteria isolated from jujube *Ziziphus lotus* plant stimulate wheat germination rate and seedlings growth. *PeerJ*, *9*, e11583 https://doi.org/10.7717/peerj.11583

Goswami, D., Vaghela, H., Parmar, S., Dhandhukia, P., & Thakkera, J. N. (2013). Plant growth promoting potentials of *Pseudomonas* spp. strain OG isolated from marine water. *Journal of Plant Interactions*, *8*(4), 281-290. https://doi.org/10.1080/17429145.2013.768360

Haegele, T., Bunnom, T., Khumhom, S., Braeuchler, C., Liplap, P., & Arjharn, W. (2017). Expanding the farming potential of Napier grass (*Pennisetum purpureum* SCHUMACH.) under low-fertile conditions. *Suranaree Journal of Science and Technology*, *24*(2), 137-151.

Huang, X., El-Alawi, Y., Penrose, D. M., Glick, B. R., & Greenberg, B. M. (2004). Response of three grass species to creosote during phytoremediation. *Environmental Pollution*, *130*(3), 453-363. https://doi.org/10.1016/j.envpol.2003.12.018

Khan, N., Bano, A., & Babar, M. A. (2017). The root growth of wheat plants, the water conservation and fertility status of sandy soils influenced by plant growth promoting rhizobacteria. *Symbiosis*, *72*(3), 195-205. https://doi.org/10.1007/s13199-016-0457-0

Kumar, A., Singh, S., Gaurav, A. K., Srivastava, S., & Verma, J. P. (2020). Plant growth-

promoting bacteria: Biological tools for the mitigation of salinity stress in plants. *Frontiers in Microbiology*, *11*, 1216. https://doi.org/10.3389/fmicb.2020.01216

Kumar, B. L., & Gopal, D. V. R. S. (2015). Effective role of indigenous microorganisms for sustainable environment. *3 Biotech*, *5*, 867-876. https://doi.org/10.1007/s13205-015-0293-6

Lakshminarayanan, V., Ponnuswamy, R., & Rengaraju, B. (2015). Screening, purification and characterization of ß-glucan from a novel strain *Bacillus cereus* LVK13 (KC 898956). *International Journal of ChemTech Research*, *8*(3), 1156-1162.

Li, X., Geng, X., Xie, R., Fu, L., Jiang, J., Gao, L., & Sun, J. (2016). The endophytic bacteria isolated from elephant grass (*Pennisetum purpureum* Schumach) promote plant growth and enhance salt tolerance of hybrid *Pennisetum*. *Biotechnology for Biofuels*, *9*, 190. https://doi.org/10.1186/s13068-016-0592-0

Machado, S., & Paulsen, G. M. (2001). Combined effects of drought and high temperature on water relations of wheat and sorghum. *Plant and Soil*, *233*(2), 179-187. https://doi.org/10.1023/A:1010346601643

Mei, C., Amaradasa, S., Sikaroodi, M., Zhang, X., Gillevet, P., Nowak, J., & Lowman, S. (2021). Chapter 7 - Potential application of plant growth promoting bacteria in bioenergy crop production. In J. White, A. Kumar, & S. Droby (Eds.), *Microbiome stimulants for crops* (pp. 109-123). Woodhead Publishing. https://doi.org/10.1016/B978-0-12-822122-8.00014-5

Nantasaksiri, K., Charoen-Amornkitt, P., & Machimura, T. (2021). Land potential assessment of Napier grass plantation for power generation in Thailand using SWAT model. Model validation and parameter calibration. *Energies*, *14*(5), 1326. https://doi.org/10.3390/en14051326

Negawo, A. T., Teshome, A., Kumar, A., Hanson, J., & Jones, C. S. (2017). Opportunities for Napier grass (*Pennisetum purpureum*) improvement using molecular genetics. *Agronomy*, *7*(2), 28. https://doi.org/10.3390/agronomy7020028

Niu, S., Gao, Y., Zi, Z., Liu, Y., Liu, X., Xiong, X., Yao, Q., Qin, Z., Chen, N., Guo, L., Yang, Y., Qin, P., Lin, J., & Zhu, Y. (2022). The osmolyte-producing endophyte *Streptomyces albidoflavus* OsiLf-2 induces drought and salt tolerance in rice via a multi-level mechanism. *The Crop Journal, 10*(2), 375-386. https://doi.org/10.1016/j.cj.2021.06.008

Odiyi, B. O., & Oludare, P. A. (2015). Impact of simulated salinity gradient on growth indices of *Pennisetum purpureum* Schumach. *Jordan Journal of Agricultural Sciences*, *11*(3), 661-667. https://journals.ju.edu.jo/JJAS/article/view/10315/4651

Osman, N. A., Roslana, A. M., Ibrahima, M. F., & Hassana M. A. (2020). Potential use of *Pennisetum purpureum* for phytoremediation and bioenergy production: A mini review. *Asia Pacific Journal of Molecular Biology and Biotechnology*, *28*(1), 14-26. https://doi.org/10.35118/apjmbb.2020.028.1.02

Pereira, N. C. M., Galindo, F. S., Gazola, R. P. D., Dupas, E., Rosa, P. A. L., Mortinho, E. S., & Teixeira Filho, M. C. M. (2020). Corn yield and phosphorus use efficiency response to phosphorus rates associated with plant growth promoting bacteria. *Frontiers in Environmental Science*, *8*, 40. https://doi.org/10.3389/fenvs.2020.00040

Sade, N., Galkin, E., & Moshelion, M. (2015). Measuring *Arabidopsis*, tomato and barley leaf relative water content (RWC). *Bio-Protocol*, *5*(8), e1451. https://doi.org/10.21769/BioProtoc.1451

Sangdee, A., Kornphachara, S., & Srisawat, N. (2016). *In vitro* screening of antagonistic activity of soil *Streptomyces* against plant pathogenic fungi and assessment of its characters. *International*

*Journal of Agricultural Technology*, *12*(1), 173-185.

Somtrakoon, K., Sabutong, B., Srinoi, P., Chaiyasit, R., Sangdee A., & Chouychai W. (2021). Pattern of *Streptomyces* sp. culture filtrate application on seedling growth of rice cv. RD6 cultivated under fluorene or phenanthrene contamination. *Journal of Agricultural Research and Extension*, *38*(3), 15-27.

Somtrakoon, K., Sangdee, A., Chouychai, W. (2019a). Roles of plant growth promoting bacteria on growth of ornamental plants grown in anthracene-spiked soil. *Journal of Agricultural Research and Extension*, *36*(2), 11-22.

Somtrakoon, K., Sripasa, N., Ladsena, S., Sangdee, A., & Chouychai, W. (2019b). Optimum conditions for indole-3-acetic acid production by *Streptomyces* and its stimulation on seed germination of rice cv. KDML105. *Journal of Agricultural Research and Extension*, *36*(3), 12-22.

Videira, S. S., de Oliveira, D. M., de Morais, R. F., Borges, W. L., Baldani, V. L. D., & Baldani, J. I. (2012). Genetic diversity and plant growth promoting traits of diazotrophic bacteria isolated from two *Pennisetum purpureum* Schum. genotypes grown in the field. *Plant Soil*, *356*, 51-66. https://doi.org/10.1007/s11104-011-1082-6

Waramit, N., & Chaugool, J. (2014). Napier grass: A novel energy crop development and the current status in Thailand. *Journal of the International Society for Southeast Asian Agricultural Sciences*, *20*(1), 139-150.

Xu, Z., Mei, X., Tan, L., Li, Q., Wang, L., He, B., Guo, S., Zhou, C., & Ye, H. (2018). Low root/shoot (R/S) biomass ratio can be an indicator of low cadmium accumulation in the shoot of Chinese flowering cabbage (*Brassica campestris* L. ssp. *chinensis* var. *utilis* Tsen et Lee) cultivars. *Environmental Science and Pollution Research*, *25*, 36328–36340. https://doi.org/10.1007/s11356-018-3566-x

Yandigeri, M. S., Meena, K. K., Singh, D., Malviya, N., Singh, D. P., Solanki, M. K., Yadav, A. K., & Arora, D. K. (2012). Drought-tolerant endophytic actinobacteria promote growth of wheat (*Triticum aestivum*) under water stress conditions. *Plant Growth Regulation*, *68*, 411-420. https://doi.org/10.1007%2Fs10725-012-9730-2

Zhang, S., Xu, X., Sun, Y., Zhang, J., & Li, C. (2018). Influence of drought hardening on the resistance physiology of potato seedlings under drought stress. *Journal of Integrative Agriculture*, *17*(2), 336–347. https://doi.org/10.1016/S2095-3119(17)61758-1

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# Performance and *In vivo* Digestibility of Three Varieties of Napier Grass in Thin-Tailed Sheep

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## ABSTRACT

This study aimed to determine the effect of grass variety on intake, nutrient digestibility, and performance of thin-tailed sheep. The research was conducted in Suket Ijo Farm, Sanggrahan, Wedomartani, Sleman, Yogyakarta. Twelve female thin-tailed sheep with an average body weight of 15 kg and the age of 8 to 10 months were used in this study. The sheep were given the feed formulation based on dry matter (DM): (67%), water spinach straw (8%), and 25% of either Gamma Umami grass (P1), local Napier grass (P2), or dwarf Napier grass (P3). The variables observed were feed nutrient consumption, nutrient digestibility, and thin-tailed sheep performance. The data obtained were analyzed using analysis of variance (ANOVA), and the means were separated using Duncan's Multiple Range Test (DMRT). The results showed that the treatment feed had a significant effect ($P<0.05$) on the consumption of dry matter (DM), organic matter (OM), crude fiber (CF), dry matter digestibility (DMD), organic matter digestibility (OMD), crude protein digestibility (CPD), crude fiber digestibility (CFD), average daily gain (ADG), and ration conversion. However, it had no significant effect ($P>0.05$) on crude protein (CP) consumption and extract ether digestibility (EED). The highest ADG was in treatment P1, 105.46 g, with a ration conversion of 5.74. Hence, it was concluded

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that the diet containing Napier grass variety Gamma Umami showed higher feed nutrient digestibility and improved thin-tailed sheep's performance.

*Keywords*: *In vivo*, Napier grass, performance, thin-tailed sheep

## INTRODUCTION

Feed plays an important role in the ruminant livestock business. The feed contains various nutrients needed by livestock both for basic life, production, and reproduction. The ruminant feed consists of two types, namely concentrate and forage. The concentrate is a feed with a high nutritional value, is easy to digest, and contains various feed ingredients. Forage is a feed containing fiber needed for the fermentation digestion in the rumen. Ruminants generally consume forage types of grass. One popular grass used by breeders is Napier grass (*Pennisetum purpureum*).

Napier grass originally comes from Africa, and it can be adapted to various conditions. Ananta et al. (2019) stated that Napier grass is superior because it can grow well on poor soil. It also has high productivity and good nutrient content to meet the livestock needs. Pre-study data showed that Napier grass contains 19.162% dry matter (DM), 86.07% organic matter (OM), 8.19% crude protein (CP), 3.09% extract ether (EE), and 32.70% crude fiber (CF).

However, Napier grass has problems in its development as an animal feed. Therefore, an improvement in feed quality does not accompany the increasing livestock population. The limitation on the Napier grass quality is a factor that hinders the fulfillment of the nutritional needs of animal feed. Fahmi et al. (2019) stated that Napier grass contains high fiber and low extract without nitrogen. Napier grass is a C4 plant with high productivity characteristics but is not supported by good quality. Therefore, efforts are needed to increase the productivity and quality of Napier grass. One solution to improve the quality of Napier grass is selection and mutation.

The selection of Napier grass varieties aims to obtain better quality. One of the best varieties is *Pennisetum purpureum* cv. Mott. Ananta et al. (2019) reported that dwarf grass contains dry matter and crude protein of 13.96% and 12.58%, respectively. However, this forage has low productivity (Utomo et al., 2020).

Mutation breeding can be done to increase the productivity of Napier grass quality. Mutation breeding uses mutation induction to develop better varieties (Chahal & Gosal, 2003). The mutation process will create changes in the genetic material of an organism. Therefore, changes in the mutation process can increase diversity which is expected to improve plant quality. One of Napier grass varieties resulting from mutation breeding is *Pennisetum purpureum* cv. GU (Gamma Umami grass).

Gamma Umami grass is a new variety of Napier grass developed by Universitas Gadjah Mada in 2018. Gamma Umami grass is derived from conventional Napier grass, which is mutated by radiation gamma with a wavelength of 100 Gy. This grass is a grass

collection from the Forage Farm, Faculty of Animal Science, Universitas Gadjah Mada. This variety of Napier grass contains 20.55% of DM, 85.54% of OM, 10.76% of CP, 32.50% of CF, and 1.33% of EE under the planting conditions without fertilization.

Gamma Umami grass, local Napier grass, and dwarf Napier grass have different characteristics and potential to be developed as animal feed. However, the three varieties of Napier grass have their respective advantages and disadvantages, so they need to be investigated to obtain more information as ruminant feed. Therefore, the study was conducted on three Napier grass varieties' performance and *in vivo* digestibility in thin-tailed sheep.

## MATERIALS AND METHODS

The *in vivo* digestibility trial was carried out at the Suket Ijo Farm, Sanggrahan, Wedomartani, Sleman, Yogyakarta. In contrast, the proximate analysis for dry matter (DM), organic matter (OM), crude protein (CP), and crude fiber (CF) on feed samples, feed residue, and feces was carried out at the Forage and Pasture Science Laboratory, Faculty of Animal Science, Universitas Gadjah Mada.

### Ethical Approval

The ethical eligibility commission approved this study protocol for pre-clinical trials (No.0051/EC-FKH-Eks./2020) from the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.

### Animal

The animal used as the object of this study was female thin-tailed sheep (N = 12) aged 8-10 months with an average body weight of 15 kg. The animal was placed in metabolism crates with dimensions 70 x 150 cm and had continuous access to freshwater (*ad libitum*). Each metabolic pen is equipped with a separate fecal and urine collecting bucket. The study applied the deworming Leva-200® (Indonesia) oral at a 1 cc/20kg body weight dosage to remove worm infection during the research (Rahayu et al., 2021). Hair removal was also carried out before the study. Shearing aimed to get the net body weight and avoid heat stress during maintenance.

### Diets

The sheep were given diets containing (DM) concentrates (67%), water spinach straw (8%), and 25% of either Gamma Umami grass (P1), local elephant grass (P2), or dwarf Napier grass (P3) (Table 2). In this study, the feed was given *ad libitum*. Feed was given twice daily at 8 a.m. and 4 p.m. Feeding of commercial concentrate JF49® (Indonesia) and water spinach straw was mixed, while Napier grass was given *ad libitum* for the next 1 hour.

### *In vivo* Digestibility

**Adaptation Period.** The adaptation period was carried out for 14 days. Feed was given twice, namely at 8 a.m. and at 4 p.m. The drinking water was given *ad libitum* (Wulandari et al., 2014).

**Period of Maintenance, Collection, and Analysis of Samples.** Samples maintenance, collection, and analysis followed Wulandari et al. (2014) with modifications. First, maintenance was carried out for six weeks. Next, the initial weight was determined based on weighing at the end of the adaptation period, followed by every two weeks to minimize stress (Purnamasari et al., 2021). Finally, the feed residue was taken and weighed to determine consumption.

The collection of feed and feces was held for ten days before the end of the study. Feed collection was done by weighing the feed and leftover feed. The feces collection was carried out every morning before the sheep were fed. The feed and feces samples were sampled for proximate analysis (Association of Official Analytical Chemists [AOAC], 2005). The fiber fraction was measured using the method of Van Soest et al. (1991). The measured fiber fraction consisted of neutral detergent fiber (NDF) and acid detergent fiber (ADF).

## Research Design and Data Analysis

The study used a completely randomized design with a one-way pattern with three treatments and four replications. The variables studied included nutrient consumption (DM, OM, CP, and CF), nutrient digestibility (DMD, OMD, CPD, and CFD), average daily gain (ADG), and ration conversion. The data obtained were analyzed by analysis of variance using software Statistical Product and Service Solution (SPSS) version 20. Further testing was carried out with the Duncan's New Multiple Range Test (DMRT) to significantly different data.

Table 1

*Nutrient content (%) of feed ingredients*

| Materials | *Pennisetum purpureum* cv. GU | Local Napier grass | Dwarf Napier grass | JF49® concentrate | Water spinach straw |
|---|---|---|---|---|---|
| Dry matter | 20.55 | 19.62 | 12.61 | 90.6 | 88.56 |
| Crude protein | 10.76 | 8.19 | 13.32 | 18.13 | 6.28 |
| Crude fiber | 32.5 | 32.7 | 25.77 | 10.97 | 29.18 |
| Extract ether | 1.33 | 3.09 | 1.37 | 6.94 | 2.45 |
| Ash | 14.46 | 13.94 | 19.19 | 7.94 | 14.82 |
| Nitrogen free extract | 40.95 | 42.08 | 40.34 | 56.02 | 42.49 |
| Total digestible nutrients | 53.75 | 54.82 | 59.52 | 73.39 | 56.39 |
| Neutral detergent fiber | 66.65 | 75.94 | 65.93 | - | - |
| Acid detergent fiber | 36.65 | 40.28 | 42.06 | - | - |

Source: Analysis results from forage and Pasture Laboratory, Faculty of Animal Science, Universitas Gadjah Mada

Table 2

*The proportion of feed ingredients (%) and nutrient content (%) of ration treatment*

| | P1 | P2 | P3 |
|---|---|---|---|
| Materials | | | |
| *Pennisetum purpureum* cv. GU | 25 | 0 | 0 |
| Local Napier grass | 0 | 25 | 0 |
| Dwarf Napier Grass | 0 | 0 | 25 |
| Water spinach straw | 8 | 8 | 8 |
| JF49® concentrate | 68 | 68 | 68 |
| Nutrient content | | | |
| Dry matter (DM) | 72.92 | 72.69 | 70.94 |
| Organic matter (OM) | 89.88 | 90.01 | 88.70 |
| Crude protein (CP) | 15.34 | 14.70 | 15.98 |
| Crude fiber (CF) | 17.81 | 18.86 | 16.13 |
| Extract ether (EE) | 5.18 | 5.62 | 5.19 |

*Note*. P1 = Concentrate + gamma grass; P2 = Concentrate + local Napier grass; P3 = Concentrate + dwarf Napier grass

## RESULTS AND DISCUSSION

### Nutrient Consumption

The treatment feed had a significant effect ($P$<0.05) on nutrient consumption. Different feed treatments affected the consumption of DM, OM, and CF but did not affect the consumption of CP (Table 3).

Table 3

*Nutrient consumption of thin-tailed sheep fed with different ration treatment*

| Nutrient consumption (g/head/day) | Treatment | | |
|---|---|---|---|
| | P1 | P2 | P3 |
| Dry matter (DM) | 601.20±23.70$^{a}$ | 575.16±5.35$^{ab}$ | 542.44±31.63$^{b}$ |
| Organic matter (OM) | 541.61±21.30$^{a}$ | 520.05±4.81$^{a}$ | 486.71±28.04$^{b}$ |
| Crude protein (CP)$^{ns}$ | 90.22±2.65 | 83.98±0.72 | 86.76±4.91 |
| Crude fiber (CF) | 112.48±8.65$^{a}$ | 103.23±1.14$^{a}$ | 86.02±5.63$^{b}$ |
| Extract ether (EE) | 29.71±0.87$^{a}$ | 32.23±0.27$^{b}$ | 28.73±1.48$^{a}$ |

*Note*. $^{ab}$Different superscripts on the same row showed significant differences ($P$<0.05)
P1 = Concentrate + gamma grass; P2 = Concentrate + local Napier grass; P3 = Concentrate + dwarf Napier grass

The differences in the nutrient content of the ration affected dry matter consumption. Based on Table 1, Gamma Umami grass contained higher dry matter (20.55%) compared to local Napier grass (19.62%) and dwarf Napier grass (12.61%). The high dry matter content of Gamma Umami grass caused the dry matter consumption of P1 treatment to increase in the same amount of as-feed consumption. Nurjannah et al. (2019) stated that dry matter consumption would determine the number of nutrients that enter the livestock body.

In this study, dry matter consumption was 542.44 to 601.20 g/head/day. The value of dry matter consumption in this study was lower than the research of Wulandari et al. (2014), which used thin-tailed sheep fed by complete feed and supplemented with Napier grass and cocoa pods as a source of fiber with a dry matter consumption was 970.8 to 1.008.3 g/head/day. The results of this study were also lower than the research of Audhar et al. (2020), which used thin-tailed sheep fed by concentrate with the addition of Napier grass, field grass, and *Leucaena leucocephala* as a source of fiber with a dry matter consumption was 912.26 to 959.28 g/head/day.

The crude protein intakes were not significantly different among the dietary treatments, possibly due to dry matter intake. Riyanto et al. (2020) stated that CP consumption is closely related to DM consumption. Yulianti et al. (2019) added that the consumption of crude protein is strongly influenced by the nutritional content of crude protein in the ration. Dry matter consumption in treatment P1 was higher than in treatment P3 and was not significantly different from P2. In contrast, the protein content in the ration P3 treatment was the highest (15.98%) compared to P1 (15.34%) and P2 (14.70%) (Table 2). The high consumption of dry matter in treatments P1 and P2 was not supported by the high crude protein content of the rations, while the P3 treatment rations contained higher crude protein but had lower dry matter consumption. It causes the consumption of crude protein to be not significantly different. The NDF values in P1 and P3 were similar, so the flow rate in the rumen of the sheep fed diet P1 and P3 was similar. Feed flow affects feed consumption and nutrient content. The higher the feed flow, the faster emptying the rumen contents, stimulating livestock to consume the feed. Pino et al. (2018) reported that the NDF content of the feed was positively correlated with the rumen fluid flow rate. Almeida et al. (2019) reported that the proportion of each cell wall component influences the nutrient intake.

The crude fiber consumption level in the ration was positively correlated with dry matter and organic matter consumption. Treatment P3 had lower dry matter consumption than P1 and P2, which happened to crude fiber consumption (Table 3). It was because crude fiber is a part of dry matter, which is influenced by dry matter consumption. The low crude fiber content in the P3 treatment was also the cause of the low consumption of crude fiber. Kamalidin et al. (2012) reported that different fiber content and DM consumption in feed were

some of the factors that determined fiber consumption.

Extracting ether content in the ration affected the consumption of extract ether. Table 1 showed that the extract ether content of local Napier grass was higher (3.09%) than Gamma Umami grass (1.33%) and dwarf Napier grass (1.37%). Table 3 showed that the highest extract ether consumption was in the P2 treatment. The high extract ether content in local Napier grass causes an increase in extract ether consumption in P2 treatment. Kamalidin et al. (2012) reported that the high consumption of extract ether was caused by an increase in the extract ether content of the feed.

**Nutrient Digestibility**

The treatment feed had a significant effect ($P$<0.05) on nutrient digestibility. Different feed treatments affected the digestibility of DM, OM, CP, and CF (Table 4).

The low dry matter digestibility in the P2 treatment was due to differences in the feed nutrient content. Based on Table 2, the P2 treatment feed had the lowest protein content compared to other treatments. Protein is a food source for rumen microbes because it contains nitrogen (N). Microbes will utilize N and carbohydrates to grow and increase their population. Microbes play a role in the digestion of fermentation in the rumen. The low protein content in P2 treatment can reduce the microbial population and slow down the rumen's digestive process. Prihartini et al. (2011) explained that the factor that affects digestibility is the availability of nutrients as food for microbial growth. Suardin et al. (2015) reported that feed digestibility was influenced by the fermentation activity carried out by rumen microbes. The P2 treatment feed contained the highest crude fiber compared to the other treatment feeds. The fiber content in the feed affects the digestibility of the feed. Fiber is a component that is difficult to digest because fiber has a cell wall layer that bacteria can only degrade in the rumen. The high fiber content in P2 treatment could slow bacteria to digest fiber, decreasing digestibility. Tillman et al. (1989) stated that the more crude fiber contained in a feed ingredient, the thicker the cell wall and, consequently, the lower the digestibility of the food material. Gultom et al.'s (2016) research results showed that crude fiber content negatively correlated with digestibility.

The digestibility of organic matter decreased as increasing of the dry matter digestibility. Organic matter digestibility was positively correlated with dry matter digestibility based on these data. Dry matter digestibility projects organic matter digestibility so that when dry matter digestibility increases, organic matter digestibility also increases. Suwignyo et al. (2016) reported that organic matter digestibility was closely related to dry matter digestibility.

The high crude protein digestibility value in the P1 and P3 treatments was due to crude fiber digestibility and organic matter digestibility. Table 4 shows that the digestibility of the P1 and P3 treatments was higher than that of P2, as well as the digestibility of crude fiber and organic

Table 4

*Nutrient digestibility coefficient and performance of thin-tailed sheep fed with different ration treatment*

| | Treatment | | |
|---|---|---|---|
| | P1 | P2 | P3 |
| Nutrient Digestibility (%) | | | |
| Dry matter (DM) | 71.14±2.95[a] | 64.04±2.03[b] | 70.11±4.40[a] |
| Organic matter (OM) | 73.08±2.93[a] | 66.91±1.81[b] | 73.36±3.26[a] |
| Crude protein (CP) | 77.06±2.76[a] | 69.30±3.63[b] | 75.67±2.75[a] |
| Crude fiber (CF) | 57.99±4.81[a] | 48.29±2.16[b] | 54.33±4.80[ab] |
| Extract ether (EE) [ns] | 86.23±10.97 | 81.45±22.83 | 94.40±1.31 |
| Performance | | | |
| ADG (g/head/day) | 105.48±13.25[a] | 84.65±7.36[b] | 87.05±11.18[b] |
| Ration conversion | 5.74±0.45[a] | 6.83±0.58[b] | 6.27±46[ab] |

*Note*. [ab]Different superscripts on the same row showed significant differences ($P$<0.05)
P1 = Concentrate + gamma grass, P2 = Concentrate + local Napier grass, P3 = Concentrate + dwarf Napier grass

matter. Crude fiber is a nutrient component with strong chemical bonds, so it is difficult to be degraded by rumen microbes. Fiber digestibility affects the digestibility of other nutrients because some nutrients bind to fiber. Proteins bound to the cell wall will not be digested before the cell wall undergoes a degradation process. The increased digestibility of crude fiber at P1 and P3 increased the digestibility of crude protein. It was in line with Wulandari et al. (2014), where an increase influences the increased digestibility value in the amount of digested crude fiber. Crude protein digestibility is also positively correlated with organic matter digestibility because crude protein is part of organic matter. Table 4 shows that the digestibility of organic matter in the P1 and P3 treatments is higher than in P2 and the digestibility of crude protein. Somanjaya et al. (2016) reported that the digestibility of organic matter is related to the digestibility of crude protein.

The different crude fiber content in the treatment rations was thought to cause the low crude fiber digestibility in P2 feed. Table 2 shows that the P2 treatment feed contained the highest crude fiber (18.86%) compared to P1 (18.81%) and P3 (16.13%). Table 4 shows that the digestibility of crude fiber in treatment P2 (48.29%) was lower than P1 (57.99%) and P3 (54.33%). It shows a negative correlation between the ration's crude fiber content and its digestibility coefficient. Crude fiber is a nutrient composition that is difficult to digest. Crude fiber contains cellulose and lignin, which rumen microbes can only digest. Rumen microbes attach to plant particles and secrete enzymes to carry out the degradation process. The high crude fiber content in the P2 treatment ration

indicated the thicker the cell wall layer due to the higher lignin and cellulose content, thus slowing the microbial penetration process into the crude fiber. Microbial penetration of the inhibited feed will reduce the level of digestibility. Somanjaya et al. (2016) reported that the digestibility of crude fiber is highly dependent on the content of crude fiber in the ration. Another factor that affects the digestibility of crude fiber is the activity of cellulolytic bacteria in the rumen. Tillman et al. (1989) added that fiber is the component that most determines digestibility because it is a building material for cell walls that is difficult to degrade.

The treatment ration did not affect extract ether digestibility. The extract ether content, which did not affect the treatment rations, could cause the extract ether digestibility to differ. Digestibility value is determined by the chemical composition of the feed constituents. Based on Table 3, it could be known that the extract ether content of the P1, P2, and P3 treatments were 5.18%, 5.64%, and 5.19%. Polii et al. (2020) stated that the same extract ether content feed ingredients had the same extract ether digestibility value.

**Livestock Performance**

The treatment feed had a significant effect ($P<0.05$) on performance. In addition, different feed treatments influenced average daily gain (ADG) and ration conversion (Table 4).

ADG is one of the factors that determine livestock performance. The higher the ADG value indicates good livestock performance. It also indicates the better quality of feed consumed by livestock. The research data shows that the P1 treatment ration gave the highest ADG value. The high ADG in P1 treatment was caused by several factors, including quality, consumption, and feed digestibility.

The value of ration consumption shows the number of nutrients consumed by livestock. The higher the consumption of the ration, the more nutrients used by livestock. Nutrients are used by livestock for basic living, production, and reproduction. The data in this study (Table 3) shows that the consumption of dry matter, organic matter, and crude fiber in the rations of P1 and P2 treatment was higher ($P<0.05$) compared to P3. It indicated that more nutrients consumed by livestock are needed to increase ADG. Purnamasari et al.'s (2021) research results showed that feed consumption was directly proportional to the increase in daily bodyweight gain.

Nutrient digestibility influences determining the ADG value. Table 4 shows that treatments P1 and P3 rations resulted in higher dry matter, organic matter, crude protein, and crude fiber digestibility than P2 treatment. The increase in nutrient digestibility indicated that the nutrient components contained were more widely used by livestock. The more nutrients are used by livestock, the more ADG increases. Hernaman et al. (2018) stated that the digestibility of feed ingredients determines sheep productivity.

The high ADG value in the P1 treatment was closely related to the ration's nutritional

value, the ration's consumption, and the ration's digestibility. Sheep fed with P1 and P2 treatments resulted in higher nutrient consumption (dry matter, organic matter, and crude fiber) than sheep fed with P3 treatment (Table 3). In addition, sheep fed with P1 and P3 treatments gave higher nutrient digestibility results (dry matter, organic matter, crude protein, and crude fiber) than sheep fed with P2 treatment (Table 4). The P1 treatment ration had nutrient consumption and high digestibility advantages based on these data. It indicates that the P1 treatment ration had good quality and palatability to provide high nutrient adequacy for livestock. Livestock absorbs the high nutrient and then uses it to increase ADG. Adiwimarta (2021) stated that the quality and quantity of rations could affect the livestock's nutritional requirement, which will affect livestock productivity.

Feed consumption was positively correlated with the growth performance of sheep. Table 3 shows that the highest dry matter consumption was in the P1 treatment. Table 4 shows that the highest ADG was found in the P1 treatment. Based on these data, it could be known that higher dry matter consumption can cause higher ADG: the higher the dry matter consumption, the more nutrients consumed by livestock. Nutrients are used by livestock to increase body weight. Tricahyani et al. (2017) reported that feed consumption is directly proportional to ADG.

The ADG value in this study was 84.65 to 105.46 g/head. The ADG value in this study was lower than the research of Wulandari et al. (2014), with the ADG value being 140.0 g/head-147.1 g/head. The results of this study were also lower than the research of Audhar et al. (2020), with the ADG value being 108.75 g/head-149.82 g/head.

Conversion of ration in P1 treatment is the lowest. ADG factors and dry matter consumption caused the low value of ration conversion in P1 treatment. The ADG value in the P1 treatment was the highest in P2 and P3 treatments (Table 4). Hence, the comparison between ration consumption and ADG in the P1 treatment had the lowest value compared to P2 and P3. The low conversion rate showed that the P1 treatment ration was the most efficient in producing the product. The smaller the ration conversion value indicates, the less ration is used to produce units of body weight gain. Nurjannah et al. (2019) stated that the ration conversion value could be influenced by the dry matter consumption of the ration and ADG. Wijaya et al. (2016) stated that low feed consumption and high ADG could increase feed efficiency value.

## CONCLUSION

The sheep-fed diets containing Gamma Umami Napier grass performed better than those fed dwarf Napier or local Napier grass. It was thought that the nutritive value of Gamma Umami Napier grass contributed to the improved ADG and digestibility of nutrients in the total diet of thin-tailed sheep. In the ration, thin-tailed sheep fed with Gamma Umami grass had the highest ADG value with the lowest conversion value.

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## REFERENCES

Adiwimarta, K. I. S. (2021). *Nutrisi ruminansia: Kepentingan energi dan protein* [Ruminant nutrition: The importance of energy and protein]. Gadjah Mada University Press.

Almeida, J. C. S., de Figueiredo, D. M., de Azevedo, K. K., Paixão, M. L., Ribeiro, E. G., & Dallago, G. M. (2019). Intake, digestibility, microbial protein production, and nitrogen balance of lambs fed with sorghum silage partially replaced with dehydrated fruit by-products. *Tropical Animal Health and Production*, *51*(3), 619–627. https://doi.org/10.1007/s11250-018-1734-0

Ananta, D., Bachruddin, Z., & Umami, N. (2019). Growth and production of 2 cultivars (*Pennisetum purpureum* Schumach.) on regrowth phase. In *IOP Conference Series: Earth and Environmental Science* (Vol. 387, No. 1, p. 012033), IOP Publishing. https://doi.org/10.1088/1755-1315/387/1/012033

Association of Official Analytical Chemists. (2005). *Official methods of analysis of the AOAC International.* AOAC.

Audhar, N., Rachmadi, D., & Asril, A. (2020). Performa domba ekor tipis jantan yang diberi limbah sereh wangi (*Cymbopogon nardus*) amoniasi dengan persentase yang berbeda sebagai pengganti sebagian pakan basal [The performance of male thin-tailed sheep given fragrant lemongrass waste (*Cymbopogon nardus*) ammonia with different percentages in place of partial basal feed]. *Jurnal Ilmiah Mahasiswa*, *5*(1), 234-240. https://doi.org/10.17969/jimfp.v5i1.13763

Chahal, G., & Gosal, S. (2003). *Principles and procedures of plant breeding: Biotechnological and conventional approaches*. Narosa Publishing.

Fahmi, M., Utomo, R., & Umami, N. (2019). Physical and chemical quality of silage from two *Pennisetum purpureum* sp. varieties supplemented with molasses at different levels. In *IOP Conference Series: Earth and Environmental Science* (Vol. 387, No. 1, p. 012059). IOP Publishing. https://doi.org/10.1088/1755-1315/387/1/012059

Gultom, E. P., Wahyuni, T. H., & Tafsin, M. (2016). Kecernaan serat kasar dan protein kasar ransum yang mengandung pelepah daun kelapa sawit dengan perlakuan fisik, biologis, kimia dan kombinasinya pada domba [Digestibility of coarse fibers and coarse protein rations containing palm leaf fronds with physical, biological, chemical and combination treatment in sheep]. *Jurnal Peternakan Integratif*, *4*(2), 193–202. https://doi.org/10.32734/jpi.v4i2.2795

Hernaman, I., Ayuningsih, B., & Ramdani, D. (2018). Perbandingan model pendugaan total digestible nutrient (TDN) dan protein tercerna pada domba garut betina [Comparison of models of total digestible nutrient (TDN) and undigested protein in female scratch sheep]. *Majalah Ilmiah Peternakan*, *21*(3), 110. https://doi.org/10.24843/mip.2018.v21.i03.p04

Kamalidin, Agus, A., & Budisatria, I. G. S. (2012). Performa domba yang diberi complete feed kulit buah kakao terfermentasi [The performance of sheep given a complete feed of fermented cocoa fruit peel]. *Bulletin Peternakan*, *36*(3), 162–168. https://doi.org/10.21059/buletinpeternak.v36i3.1624

Nurjannah, S., Ayuningsih, B., Hernaman, I., & Susilawati, I. (2019). Penggunaan kaliandra (*Calliandra calothyrsus*), *Indigofera* sp. dan campurannya dalam ransum sebagai pengganti konsentrat terhadap produktivitas domba garut jantan [Use of kaliandra (*Calliandra*

*calothyrsus*), *Indigofera* sp. and the mixture in rations as a substitute for concentrate on the productivity of male scratch sheep]. *Jurnal Ilmiah Peternakan Terpadu*, *7*(3), 293-298. https://doi.org/10.23960/jipt.v7i3.p293-298

Pino, F., Mitchell, L. K., Jones, C. M., & Heinrichs, A. J. (2018). Comparison of diet digestibility, rumen fermentation, rumen rate of passage, and feed efficiency in dairy heifers fed *ad-libitum* versus precision diets with low and high quality forages. *Journal of Applied Animal Research*, *46*(1), 1296-1306. https://doi.org/10.1080/09712119.2018.1498788

Polii, D. N. Y., Waani, M. R., & Pendong, A. F. (2020). Kecernaan protein kasar dan lemak kasar pada sapi perah peranakan FH (*Friesian holstein*) yang diberi pakan lengkap berbasis tebon jagung [Digestibility of coarse protein and coarse fat in FH (*Friesian holstein*) dairy cows that are fully fed based on cornbon]. *Zootec*, *40*(2), 482-492. https://doi.org/10.35792/zot.40.2.2020.28632

Prihartini, I., Soebarinoto, Chuzaemi, S., & Winugroho, M. (2011). Karakteristik nutrisi dan degradasi jerami padi fermentasi oleh inokulum lignolitik TLiD dan BopR [Nutritient characteristics and degradation of fermented rice straw by TLiD and BopR lignolytic inoculums]. *Animal Production*, *11*(1), 1–7.

Purnamasari, L., Sari, I. W., Rahayu, S., & Yamin, M. (2021). Substitusi rumput dengan kangkung kering dan limbah tauge serta pengaruhnya terhadap performa domba garut [Substitution of grass with dried kale and bean sprout waste and its effect on the performance of scratch sheep]. *Jurnal Peternakan Indonesia*, *23*(1), 25-32. https://doi.org/10.25077/jpi.23.1.25-32.2021

Rahayu, E. R. V., Suhartanto, B., Budisatria, I. G. S., & Astuti, D. (2021). The effect of *Sorghum* varieties on digestibility and nitrogen balance of complete feed in goats. *Key Engineering Materials*, *884*, 184–190. https://doi.org/10.4028/www.scientific.net/KEM.884.184

Riyanto, J., Widyawati, S. D., & Sudibya, S. (2020). Pengaruh perbedaan rasio menir kedelai proteksi dan tanpa proteksi terhadap konsumsi, kecernaan dan nilai nutrien pakan Domba Ekor Gemuk [Effect of differences in the ratio of menir soy protection and without protection to consumption, digestibility and nutrient value of fat-tailed sheep feed]. *Livestock and Animal Research*, *18*(3), 240-245. https://doi.org/10.20961/lar.v18i3.45995

Somanjaya, R., Indah, U., Rahmah, L., & Dani, D. U. (2016). Performa dan daya cerna domba garut jantan terhadap penambahan fermentasi limbah hijauan sorgum ke dalam ransum [Performance and digestibility of male scratch sheep against the addition of fermented sorghum forage waste into the ration]. *Creative Research Journal*, *2*(2), 147–162.

Suardin, Sandiah, N., & Aka, R. (2015). Kecernaan bahan kering dan bahan organik campuran rumput mulato (*Brachiaria hybrid* cv. Mulato) dengan jenis legum berbeda menggunakan cairan rumen sapi [Digestibility of dry matter and organic matter of mulato grass mixture (*Brachiaria* hybrid cv. *Mulato*) with different types of legumes using cow rumen liquid]. *Jurnal Ilmu dan Teknologi Peternakan Tropis*, *1*(1), 16–22. https://doi.org/10.33772/jitro.v1i1.357

Suwignyo, B., Wijaya, U. A., Indriani, R., & Kurniawati, A., Widiyono, I., & Sarmin, S. (2016). Konsumsi, kecernaan nutrien, perubahan berat badan dan status fisiologis kambing Bligon jantan dengan pembatasan pakan [Consumption, digestibility of nutrients, changes in weight and physiological status of male Bligon goats with feed restrictions]. *Jurnal Sain Veteriner*, *34*(2), 210–219.

Tillman, A. D., Hartadi, H., Prawirokusumo, S. R., & Lebdosoekojo, S. (1989). *Ilmu makanan ternak dasar* [Basic fodder science]. Gadjah Mada University Press.

Tricahyani, D. N., Wulandari, S., & Nusantoro, S. (2017). Pengaruh pemberian dedak kasar fermentasi pada domba ekor tipis sebagai bahan baku konsentrat [Effect of giving fermented coarse bran on thin-tailed sheep as concentrate raw materials]. *Jurnal Ilmu Peternakan Terapan*, *1*(1), 17–24. https://doi.org/10.25047/jipt.v1i1.532

Utomo, R., Agus, A., Noviandi, C. T., Astuti, A., & Alimon, A. R. (2020). *Bahan pakan dan formulasi ransum* [Feed ingredients and ration formulations]. Gadjah Mada University Press.

Van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, *74*(10), 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Wijaya, G. H., Yamin, M., Nuraini, H., & Esfandiari, A. (2016). Performans produksi dan profil metabolik darah domba garut dan Jonggol yang diberi limbah tauge dan omega-3 [Performanceans production and metabolic profile of the blood of scratched sheep and Jonggol given bean sprout waste and omega-3]. *Jurnal Veteriner*, *17*(2), 246–256. https://doi.org/10.19087/jveteriner.2016.17.2.246

Wulandari, S., Agus, M., Soejono, M. N., Cahyanto, R., & Utomo. (2014). *Peningkatan nilai cerna serat dan penurunan theobromin pod kakao sebagai bahan baku complete feed pada domba* [Increased digestibility value of fiber and decrease in theobromin cocoa pod as raw material for complete feed in sheep] [Unpublish Doctoral dissertation]. Universitas Gadjah Mada.

Yulianti, G., Dwatmadji, D., & Suteky, T. (2019). Kecernaan protein kasar dan serat kasar kambing peranakan etawa jantan yang diberi pakan fermentasi ampas tahu dan bungkil inti sawit dengan imbangan yang berbeda [Digestibility of coarse protein and coarse fiber of goats peranakan etawa male who are fed fermented tofu pulp and palm kernel meal with different balances]. *Jurnal Sain Peternakan Indonesia*, *14*(3), 272–281. https://doi.org/10.31186/jspi.id.14.3.272-281

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# *In silico* Comparisons of the *Ethylene Response Factor 1* (*ERF1*) Gene Between Malaysian Wild Banana (*Musa acuminata* ssp. *malaccensis*) and Pisang Klutuk Wulung (*Musa balbisiana*)

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## ABSTRACT

*Musa balbisiana* (B genome) has been observed to have a higher tolerance of biotic and abiotic stresses than *Musa acuminata* (A genome). *Ethylene Response Factor 1* (*ERF1*) is a gene activator for pathogenesis-related proteins (PR proteins) such as basic chitinases and beta-1,3-glucanase. There are numerous *ERF1* gene studies about *Oryza sativa*, but information about the banana *ERF1* gene, especially in the B genome (*Musa balbisiana* "Pisang Klutuk Wulung"), has still not been explored thoroughly. Using annotated genomic data in an A genome (*Musa acuminata* ssp. *malaccensis*) and genomic data in a B genome (*Musa balbisiana* "Pisang Klutuk Wulung"), research on the *ERF1* gene can be conducted at the gene sequences and amino acid sequences levels. The *Musa acuminata* (A genome) *ERF1* gene nucleotide sequence was retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The *Musa balbisiana* (B genome) *ERF1* gene nucleotide sequence was identified with the nucleotide Basic Local Alignment Search Tool (BLASTn) using an A genome *ERF1* gene sequence as a query. Both *ERF1* gene nucleotide sequences and amino acid sequences in the A and B genomes were annotated and compared. Seven annotated genome *ERF1* gene sequences from the A and B genomes were identified with the probability that these genes were actively transcribed in cell activity. *ERF1* gene comparisons between the A and B genomes showed that nucleotide composition, gene structure, gene position in each respective chromosome, *ERF*

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clusterization, identified motif, and amino acid composition in each of the identified motifs have similar characteristics.

*Keywords*: AP2/ERF domain, comparative genomics, ethylene response factor 1, sequence annotation

## INTRODUCTION

Banana plants are commonly grown in tropical and subtropical countries. They can be classified into two groups based on their genomic composition: *Musa acuminata* (A genome) and *Musa balbisiana* (B genome) (Simmonds, 1959; Sumardi & Wulandari, 2010). The Cavendish banana cultivar (AAA) covers 90% of globally planted bananas and reduces diversity, especially at the plantation site (Drenth & Kema, 2021; Food and Agriculture Organization of the United Nations [FAO], 2019). Low diversity at plantation sites may cause problems in plants like susceptibility to a particular disease, such as the devastating Fusarium wilt (also known as "Panama Disease"), which attacked the cultivar Gros Michel (de Bellaire et al., 2010; Marín et al., 2003).

The ethylene hormone regulates the plant's defense response against the pathogen through signal transduction. The first contact in this signal transduction is the ethylene response (ETR) receptor. Next, the ETR receptor activates a signal transduction cascade by releasing the block exerted by the CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) on *Ethylene Insensitive 2* (*EIN2*) (Karlova et al., 2014). Finally, this release will actuate *EIN3/EIN3-like* (*EIL*) primary transcription factor genes (Tieman et al., 2001), leading to the activation of ethylene response factors (ERF) (Adams-Phillips et al., 2004; Bapat et al., 2010).

The ERF family, part of the AP2/ERF superfamily, is the most widely studied transcription family in plants (Riechmann & Meyerowitz, 1998). The *ERF* gene family is the gene activator for many genes (Pirrello et al., 2012). In Nakano et al. (2006), *ERF* genes were divided into groups I–X based on identified motifs besides the AP2/ERF domain. Therefore, ERF groupings based on Nakano et al. (2006) can be used as a reference to identify motifs besides AP2/ERF domain and determine the function of the sequences acquired in this study. The *ERF1* gene has been thoroughly studied in the *Arabidopsis thaliana*. It acts as a gene activator for pathogenesis-related proteins (PR proteins), such as basic-chitinases and beta-1,3-glucanase. In a previous study by Lakhwani et al. (2016), a genome-wide analysis was conducted to identify members of the AP2/ERF family in *Musa acuminata* (A genome) and *Musa balbisiana* (B genome) as well as changes leading to neofunctionalization of genes. However, information about the *ERF1* gene in the *Musa balbisiana* genome remains unexplored.

The study aimed to compare the *ERF1* genes in the A (*Musa acuminata* "DH Pahang") and B genomes (*Musa balbisiana* "Pisang Klutuk Wulung"), including their gene structure (exon-intron architecture), gene position on the chromosome, and gene function (protein clustering and motifs). Therefore, the *ERF1* gene study in *Musa*

*balbisiana* "Pisang Klutuk Wulung" can be conducted using the annotated genomic information data of *Musa acuminata* "DH Pahang" (D'Hont et al., 2012).

## MATERIALS AND METHODS

### *ERF1* Gene Nucleotide Sequences and Amino Acid Sequences Identification

A genome *ERF1* gene sequences were retrieved from KEGG (Kyoto Encyclopedia of Genes and Genomes) (https://www.genome.jp/dbgetbin/www_bget?K14516+K14517) (Kanehisa & Goto, 2000). BLAST (Basic Local Alignment Search Tool) on the banana genome hub site (https://banana-genome-hub.southgreen.fr/blast) was used to identify *ERF1* gene sequences from the B genome with the highest similarity approach (Eisen, 1998). Translated protein sequences from identified *ERF1* genes of both genomes were classified with the phylogenetic tree approach. Identified *ERF1* genes and 128 amino acid *Oryza sativa* sequences (Nakano et al., 2006) was used as a phylogenetic tree construction dataset. Phylogenetic tree construction was based on Nakano et al.'s (2006) study on the platform Molecular Evolutionary Genetics Analysis (MEGA-X) (version 10.1.5) (Kumar et al., 2018).

### *ERF1* Gene Nucleotide Comparison in *Musa acuminata* ssp. *malaccensis* and *Musa balbisiana*

Seven nucleotide sequences were retrieved and analyzed in pairs between the *MaERF1* A and B genome genes with pairwise sequence alignment and Needleman-Wunsch as the algorithm (Needleman & Wunsch, 1970) on the European Bioinformatics Institute site (EMBL-EBI) (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) (Madeira et al., 2019).

### *ERF1* Gene Structure Prediction and Visualization

Seven *ERF1* of B genome gene structures were predicted using the FGENESH+ program (Solovyev, 2007). Each predicted *ERF1* gene on both A and B genomes was visualized using the CLC Sequence Viewer (version 8.0). The location of the genes in chromosomes for both *Musa acuminata* ssp. *malaccensis* and *Musa balbisiana* were retrieved from the BLAST search and visualized using MS Paint (version 11.2201.22.0).

### ERF1 Motif Identification and Comparison in *Musa acuminata* ssp. *malaccensis*

ERF1 amino acid sequences in both *Musa acuminata* ssp. *malaccensis* and *Musa balbisiana* motifs were identified with Multiple Expectation maximizations for Motif Elicitation (MEME) suite (Bailey & Elkan, 1994) using ERF group IX consensus motifs from Nakano et al. (2006) as motif targets. The identified motif in the *Musa acuminata* ssp. *malaccensis* and *Musa balbisiana* amino acid sequences were visualized using the Weblogo3 program with default parameters (http://weblogo.threeplusone.com/create.cgi) (Crooks et al., 2004).

## RESULTS AND DISCUSSION

### *ERF1* Genes Identification in *Musa balbisiana* and *Musa acuminata*

Through searching and selections from BLASTn *ERF* gene results, seven *ERF1* genes in *Musa acuminata* (A genome) were retrieved from the KEGG database with the following gene IDs from NCBI (National Centre for Biotechnology Information): "103971653" (*MaERF1_1*), "103972093" (*MaERF1_2*), "103973681" (*MaERF1_3*), "103981246" (*MaERF1_4*), "103981564" (*MaERF1_5*), "103983138" (*MaERF1_6*), "103985947" (*MaERF1_7*). Seven *ERF1* genes in *Musa balbisiana* (B genome) were identified with gene identification: *MbERF1_1*, *MbERF1_2*, *MbERF1_3*, *MbERF1_4*, *MbERF1_5*, *MbERF1_6*, and *MbERF1_7*. *ERF1* genes in A and B genomes had a similarity of above 90% (Supplementary 1).

### *ERF1* Genes Structure and Composition in *Musa acuminata* and *Musa balbisiana*

The *ERF1* genes in *Musa acuminata* ssp. *malaccensis* and *Musa balbisiana* have no introns. The longest *ERF1* gene was *MaERF1_7* in the A genome and *MbERF1_7* in the B genome. The shortest *ERF1* gene was *MbERF1_5* in the B genome and *MaERF1_5* in the A genome (Figure 1). Like relatively short sequences, including the intron, coding sequences, and exon compared to other genes, these are the housekeeping genes' typical genomic features (Eisenberg & Levanon, 2003; Vinogradov, 2004). M. Liu et al.'s study (2019) also showed that 79.3% of *FtERF* genes had no introns. Thus, there is a probability that these *ERF1* genes are transcribed actively in cell activities. Furthermore, the nucleotide compositions of all seven *ERF1* genes in both genomes showed a similarity percentage above 95%, the data for which have been presented in Supplementary 1. These results showed that *ERF1* genes of *Musa acuminata* ssp. *malaccensis* and *Musa balbisiana* have a close evolutionary relationship because the nucleotide varieties were minimal.

### *ERF1* Genes Location in *Musa acuminata* and *Musa balbisiana* Chromosomes

The *ERF1* genes' positions in A and B genome chromosomes were similar: *ERF1_1* (for A and B genomes) in chromosome 2, *ERF1_2* in chromosome 11, *ERF1_4* and *ERF1_5* in chromosome 4, *ERF1_6* in chromosome 1, and *ERF1_7* in chromosome 5. *ERF1_3* gene in both genomes was not identified in the genome database and identified as uncategorized in the chromosome in the Banana Genome Hub database (Figure 2). Chromosome 4 has the most identified *ERF1* genes, with two in the A (*MaERF4* and *MaERF5*) and B genomes (*MbERF4* and *MbERF5*). Multiple *ERF1* genes in both banana genomes resulted from gene replication, also identified in Tartary buckwheat (*Fagopycum tataricum*) (M. Liu et al., 2019).

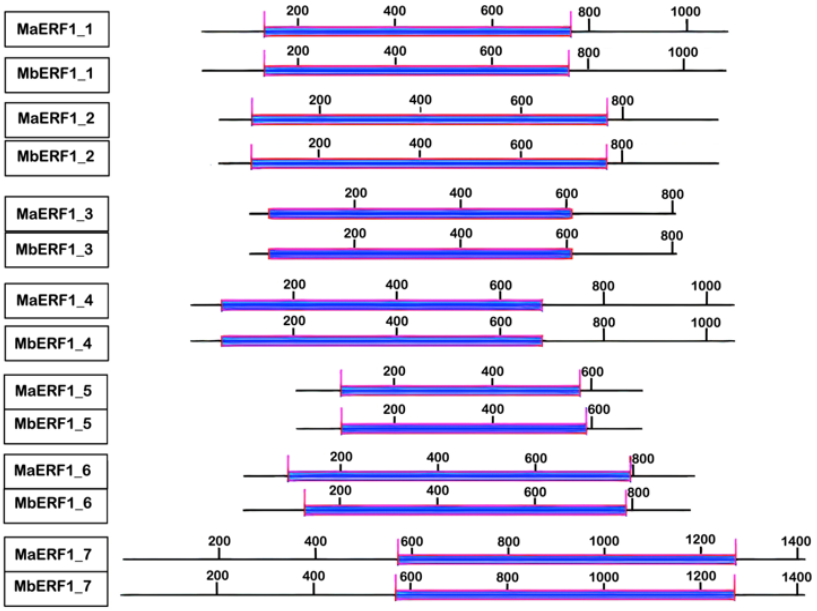


*Figure 1*. The structure of the *ERF1* gene in *Musa acuminata* (A genome) and *Musa balbisiana* (B genome). Bars are marked in the base pair (bp). The figure was visualized using the CLC Sequence Viewer (version 8.0)

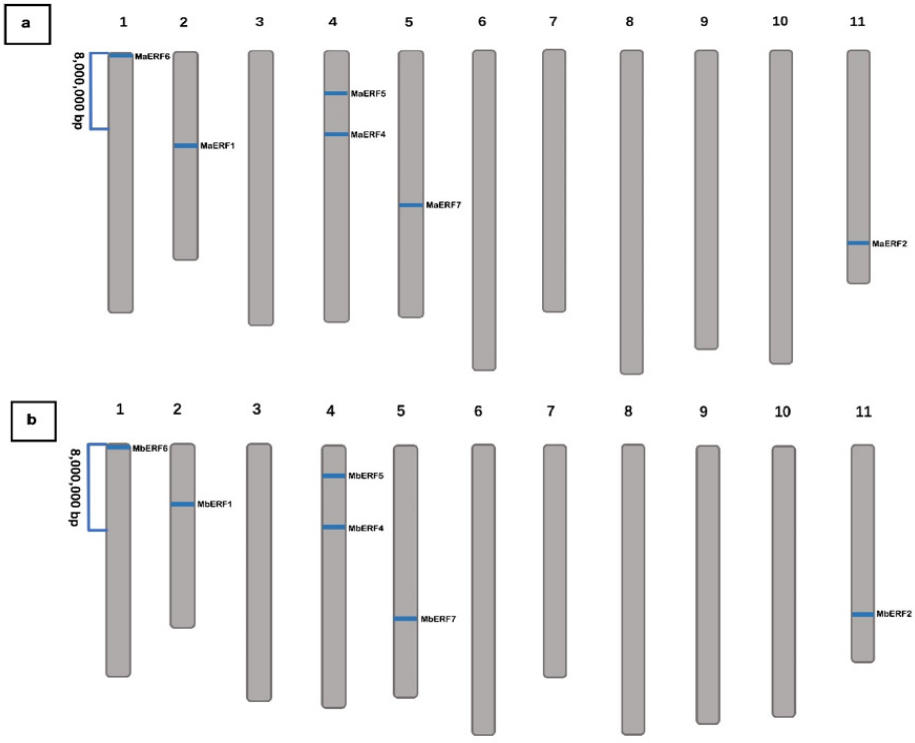


*Figure 2*. Position of *ERF1* genes in (a) *Musa acuminata* (A genome) and (b) *Musa balbisiana* (B genome) chromosomes. The figure was visualized using MS Paint (version 11.2201.22.0)

**Phylogenetic Tree Analysis**

Phylogenetic tree analysis (Figure 3) showed that MaERF1 and MbERF1 amino acid sequences (red-colored area) were in one clade with the *Oryza sativa* ERF group IX (blue-colored area). *Oryza sativa* was used as a comparison species because its *ERF* gene database was already established in a previous study by Nakano et al. (2006). *Oryza sativa* is monocotyledonous like the *Musa* species. So, based on the data, *Oryza sativa* is a widely accepted model for monocots that gives evidence of the similarities and differences between the two major groups of higher plant species and has a close lineage with *Musa* (Goff et al., 2002; Izawa & Shimamoto, 1996). The MaERF1 and MbERF1 amino acid sequences within group IX were grouped with *Oryza sativa* ERF group IXc. of the other genes, *MaERF1_1* grouped as sister taxa with *MbERF1_1*, *MaERF1_2* with *MbERF1_2*, *MaERF1_3* with *MbERF1_3*, *MaERF1_4* with *MbERF1_4*, *MaERF1_5* with *MbERF1_5*, *MaERF1_6* with *MbERF1_6*, and *MaERF1_7* with *MbERF1_7*. Phylogenetic tree analysis results showed that all *MaERF1* and *MbERF1* were ERF IXc based on ERF classification by Nakano et al. (2006), who also explained that the *ERF* gene in group IX has disease resistance roles in tomato and tobacco (Fischer & Dröge-Laser, 2004; Huang et al., 2004). *ERF1* in the A and B genomes were closely related because they are grouped as sister taxa.

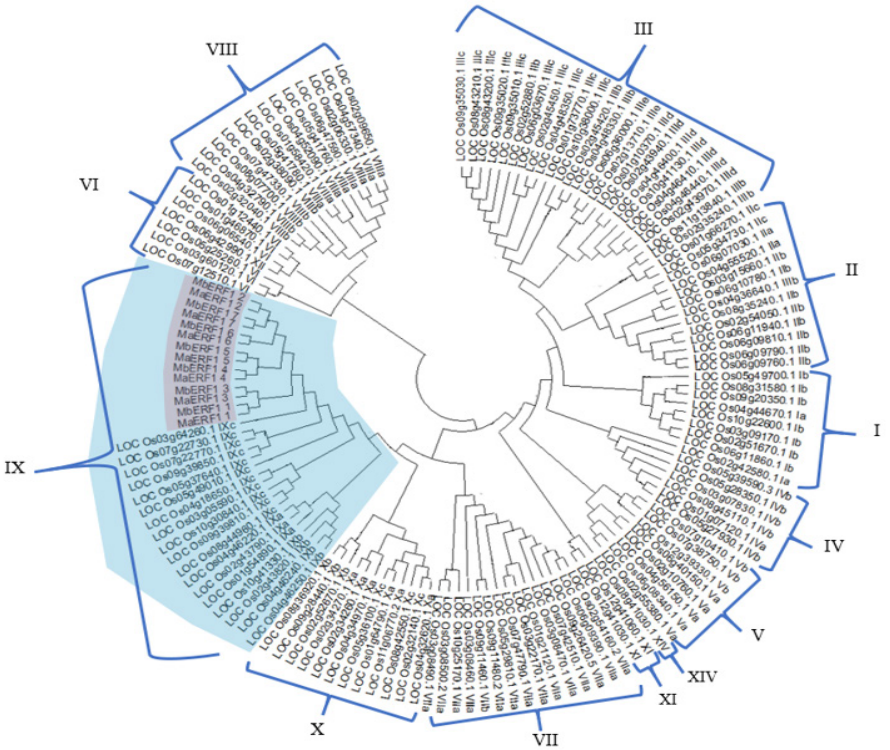


*Figure 3*. Phylogenetic tree of *Musa acuminata* (A genome), *Musa balbisiana* (B genome), and *Oryza sativa ERF* genes. The figure was constructed using MEGA-X software (version 10.1.5)

## Amino Acid Motif Composition Comparison

There were four motifs in total in the MaERF1_1, MbERF1_1, MaERF1_2, MbERF1_2, MaERF1_3, MbERF1_3, MaERF1_4, MbERF1_4, MaERF1_5, MbERF1_5, MaERF1_7, and MbERF1_7 amino acid sequences (Figure 4). There was one domain AP2/ERF (red box) and three motifs which consisted of CMIX-1 (cyan box), CMIX-3 (purple box), and CMIX-4 (orange box). In addition, three motifs were detected in MaERF1_6 and MbERF1_7. AP2/ERF, CMIX-4, and CMIX-1 were detected, while in MbERF1_6, AP2/ERF, and CMIX-4 motifs were detected. On the other hand, domain AP2/ERF was detected in all MaERF1 and MbERF1 amino acid sequences, indicating that these amino acid sequences are classified as the ERF subfamily (Riechmann & Meyerowitz, 1998). Motifs besides AP2/ERF are transcription factors likely to have similar essential functions (Rashid et al., 2012; Reyes et al., 2004) that consist of transcription factors' activities, interactions between proteins, and nuclear localization (L. Liu et al., 1999).

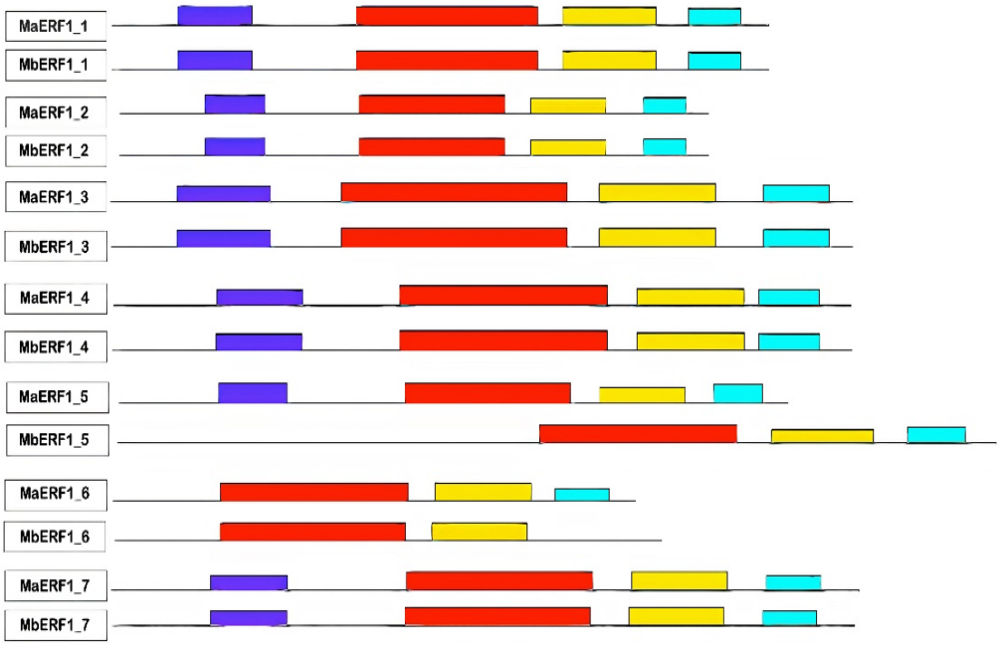


*Figure 4*. The amino acid motif of ERF sequences compares *Musa acuminata* (A genome) and *Musa balbisiana* (B genome) chromosomes. The box shows domain AP2/ERF (red box) and three motifs, which consist of CMIX-1 (cyan box), CMIX-3 (purple box), and CMIX-4 (orange box). The figure was visualized using the MEME suite program

Each identified amino acid motif of MaERF1 and MbERF1 was also analyzed. Overall, all the identified motifs between MaERF1 and MbERF1 have high similarities. For example, in Figure 6, both MaERF1 and MbERF1 have similar CMIX-4 motifs with slight amino acid composition differences detected at positions 4, 9, 19, and 22.

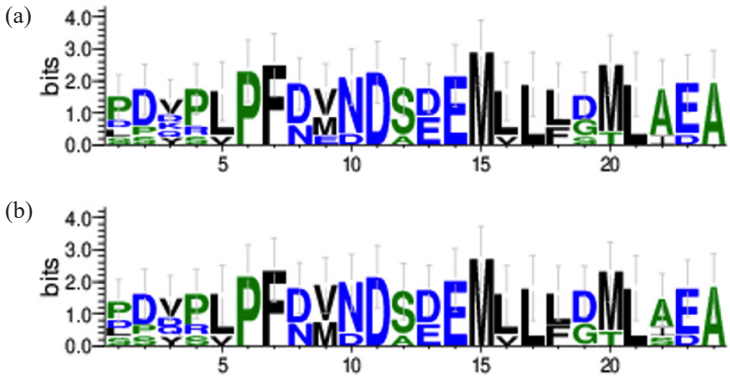


*Figure 5*. CMIX-3 motif sequences of (a) *Musa acuminata* (A genome) and (b) *Musa balbisiana* (B genome). The figure was visualized using the Weblogo3 program

In addition to the CMIX-4 motif, the amino acid composition of the CMIX-3 motif varied, but both generally have the same amino acid consensuses at each position (Figure 5). Based on the analysis, the CMIX-1 motif has the shortest amino acid sequence compared with other identified motifs, and variation between MaERF1 and MbERF1 in this motif was relatively low (Figure 7). On the other hand, the AP2/ERF motif was the longest in both genomes, with 58 amino acids. This result confirmed the previous study by Wessler (2005), which showed that AP2/ERF length was around 60–70 amino acids. Besides its length, domain motifs AP2/ERF in both genomes were conserved. There were two conserved amino acids in both genomes, YRG in position numbers 1–3 and RAYD in position numbers 39–42. YRG conserved motif was the rich basic hydrophilic amino acids located at N-terminus and has a function in DNA binding (Okamuro et al., 1997). On the other hand, the RAYD conserved domain has an essential function in domain structure and function. However, in both genomes, the AP2/ERF domain has L (leucine) in position number 39 rather than R (arginine) (Okamuro et al., 1997).

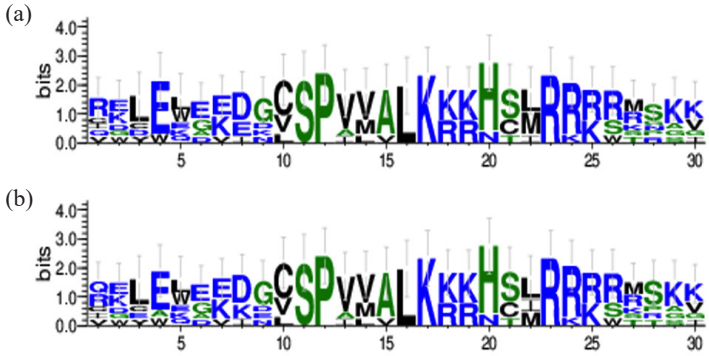


*Figure 6*. CMIX-4 motif sequences of (a) *Musa acuminata* (A genome) and (b) *Musa balbisiana* (B genome). The figure was visualized using the Weblogo3 program

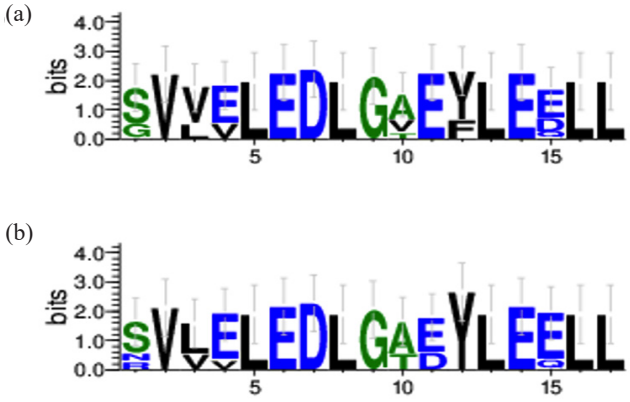


*Figure 7*. CMIX-1 motif sequences of (a) *Musa acuminata* (A genome) and (b) *Musa balbisiana* (B genome). The figure was visualized using the Weblogo3 program

Based on this study's comparison with Lakhwani et al. (2016), there were several differences in identified motifs outside the AP2/ERF domain. In Lakhwani et al. (2016), the identified motif in group IX of the *ERF* gene was only the LNFP motif. On the other hand, this study identified three motifs: CMIX-1, CMIX-3, and CMIIX-4. Furthermore, Lakhwani et al. (2016) showed that the identified motif outside AP2/ERF domain was named the LNFP motif, but based on Fujimoto et al. (2000), the LNFP motif is the part of the AP2/ERF domain amino acid residues. Considering both studies used the *in silico* approach, further confirmation is needed to elucidate the differences.

## CONCLUSION

Based on this study, the *ERF1* genes of *Musa acuminata* (A genome) and *Musa balbisiana* (B genome) showed high similarities in their nucleotide sequences, gene structures, and positions in the chromosome, phylogenetic clustering, and the motif predicted in the protein sequences.

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## AUTHORS' CONTRIBUTIONS

GK, FMD, and HN designed the study. GK analyzed the data. RRP reviewed and edited the manuscript. All authors wrote, read, and approved the final version of the manuscript.

## CONFLICT OF INTEREST

All authors declare no conflict of interest.

## REFERENCES

Adams-Phillips, L., Barry, C., & Giovannoni, J. (2004). Signal transduction systems regulating fruit ripening. *Trends in Plant Science*, *9*(7), 331–338. https://doi.org/10.1016/j.tplants.2004.05.004

Bailey, T. L., & Elkan, C. (1994). Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proceedings International Conference on Intelligent Systems for Molecular Biology*, *2*, 28–36.

Bapat, V. A., Trivedi, P. K., Ghosh, A., Sane, V. A., Ganapathi, T. R., & Nath, P. (2010). Ripening of fleshy fruit: Molecular insight and the role of ethylene. *Biotechnology Advances*, *28*(1), 94–107. https://doi.org/10.1016/j.biotechadv.2009.10.002

Crooks, G. E., Hon, G., Chandonia, J. M., & Brenner, S. E. (2004). WebLogo: A sequence logo generator. *Genome Research*, *14*(6), 1188–1190. https://doi.org/10.1101/gr.849004

de Bellaire, L. d. L., Foure, E., Abadie, C., & Carlier, J. (2010). Black leaf streak disease is challenging the banana Goff, S. A., Ricke, D., Lan, T. H., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeller, P., Varma, H., Hadley, D., Hutchison, D., Martin, C., Katagiri, F., Lange, B. M., Moughamer, T., Xia, Y., Budworth, P., Zhong, J., … Briggs, S. (2002). A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). *Science*, *296*(5565), 92–100. https://doi.org/10.1126/science.1068275

Huang, Z., Zhang, Z., Zhang, X., Zhang, H., Huang, D., & Huang, R. (2004). Tomato TERF1 modulates ethylene response and enhances osmotic stress tolerance by activating expression of downstream genes. *FEBS Letters*, *573*(1–3), 110–116. https://doi.org/10.1016/j.febslet.2004.07.064

Izawa, T., & Shimamoto, K. (1996). Becoming a model plant: The importance of rice to plant science. *Trends in Plant Science*, *1*(3), 95–99. https://doi.org/10.1016/S1360-1385(96)80041-0

Kanehisa, M., & Goto, S. (2000). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research*, *28*(1), 27–30. https://doi.org/10.1093/nar/28.1.27

Karlova, R., Chapman, N., David, K., Angenent, G. C., Seymour, G. B., & de Maagd, R. A. (2014). Transcriptional control of fleshy fruit development and ripening. *Journal of Experimental Botany*, *65*(16), 4527–4541. https://doi.org/10.1093/jxb/eru316

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Lakhwani, D., Pandey, A., Dhar, Y. V., Bag, S. K., Trivedi, P. K., & Asif, M. H. (2016). Genome-wide analysis of the AP2/ERF family in *Musa* species reveals divergence and neofunctionalisation during evolution. *Scientific Reports*, *6*, 18878. https://doi.org/10.1038/srep18878

Liu, L., White, M. J., & MacRae, T. H. (1999). Transcription factors and their genes in higher plants functional domains, evolution and regulation. *European Journal of Biochemistry*, *262*(2), 247–257. https://doi.org/10.1046/j.1432-1327.1999.00349.x

Liu, M., Sun, W., Ma, Z., Zheng, T., Huang, L., Wu, Q., Zhao, G., Tang, Z., Bu, T., Li, C., & Chen, H. (2019). Genome-wide investigation of the AP2/ERF gene family in tartary buckwheat (*Fagopyum tataricum*). *BMC Plant Biology*, *19*(1), 84. https://doi.org/10.1186/s12870-019-1681-6

Madeira, F., Park, Y. M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., Basutkar, P., Tivey, A., Potter, S. C., Finn, R. D., & Lopez, R. (2019). The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Research*, *47*(W1), W636–W641. https://doi.org/10.1093/nar/gkz268

Marín, D. H., Romero, R. A., Guzmán, M., & Sutton, T. B. (2003). Black sigatoka: An increasing threat to banana cultivation. *Plant Disease*, *87*(3), 208–222. https://doi.org/10.1094/PDIS.2003.87.3.208

Nakano, T., Suzuki, K., Fujimura, T., & Shinshi, H. (2006). Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. *Plant Physiology*, *140*(2), 411–432. https://doi.org/10.1104/pp.105.073783

Needleman, S. B., & Wunsch, C. D. (1970). A general method applicable to the search for similarities in the amino acid sequence of two proteins. *Journal of Molecular Biology*, *48*(3), 443–453. https://doi.org/10.1016/0022-2836(70)90057-4

Okamuro, J. K., Caster, B., Villarroel, R., Van Montagu, M., & Jofuku, K. D. (1997). The AP2 domain of *APETALA2* defines a large new family of DNA binding proteins in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(13), 7076–7081. https://doi.org/10.1073/pnas.94.13.7076

Pirrello, J., Prasad, B. C., Zhang, W., Chen, K., Mila, I., Zouine, M., Latché, A., Pech, J. C., Ohme-Takagi, M., Regad, F., & Bouzayen, M. (2012). Functional analysis and binding affinity of tomato ethylene response factors provide insight on the molecular bases of plant differential responses to ethylene. *BMC Plant Biology*, *12*, 190. https://doi.org/10.1186/1471-2229-12-190

Rashid, M., Guangyuan, H., Guangxiao, Y., Hussain, J., & Xu, Y. (2012). AP2/ERF transcription factor in rice: Genome-wide canvas and syntenic relationships between monocots and eudicots. *Evolutionary Bioinformatics*, *8*, EBO-S9369. https://doi.org/10.4137/EBO.S9369

Reyes, J. C., Muro-Pastor, M. I., & Florencio, F. J. (2004). The GATA family of transcription factors in *Arabidopsis* and rice. *Plant Physiology*, *134*(4), 1718–1732. https://doi.org/10.1104/pp.103.037788

Riechmann, J. L., & Meyerowitz, E. M. (1998). The AP2/EREBP family of plant transcription factors. *Biological Chemistry*, *379*(6), 633–646. https://doi.org/10.1515/bchm.1998.379.6.633

Simmonds, N. W. (1959). *Bananas*. Longman.

Solovyev, V. (2007). Statistical approaches in eukaryotic gene prediction. In D. Balding, C. Cannings, & M. Bishop (Eds.), *Handbook of statistical genetics* (3rd ed.). Wiley-Interscience. https://doi.org/10.1002/0470022620.bbc06

Sumardi, I., & Wulandari, M. (2010). Anatomy and morphology character of five Indonesian banana cultivars (*Musa* spp.) of different ploidy level. *Biodiversitas, Journal of Biological Diversity*, *11*(4), 167–175. https://doi.org/10.13057/biodiv/d110401

Tieman, D. M., Ciardi, J. A., Taylor, M. G., & Klee, H. J. (2001). Members of the tomato *LeEIL* (*EIN3-like*) gene family are functionally redundant and regulate ethylene responses throughout plant development. *The Plant Journal*, *26*(1), 47–58. https://doi.org/10.1046/j.1365-313x.2001.01006.x

Vinogradov, A. E. (2004). Compactness of human housekeeping genes: Selection for economy or genomic design?. *Trends in Genetics*, *20*(5), 248–253. https://doi.org/10.1016/j.tig.2004.03.006

Wessler, S. R. (2005). Homing into the origin of the AP2 DNA binding domain. *Trends in Plant Science*, *10*(2), 54–56. https://doi.org/10.1016/j.tplants.2004.12.007

## SUPPLEMENTARY DATA

Supplementary 1

*The sequence alignment of* ERF1 *genes in* Musa acuminata *ssp.* malaccensis *(A genome) and* Musa balbisiana *(B genome)*

```
#=======================================
#
# Aligned_sequences: 2
# 1: MaERF1_1
# 2: MbERF1_1
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 1088
# Identity:    1071/1088 (98.4%)
# Similarity:  1071/1088 (98.4%)
# Gaps:           2/1088 ( 0.2%)
# Score: 5284.5
#
#
#=======================================

MaERF1_1           1 GGTCAGACTTGCCTCGCTCTCTTTGCCTCATCCCTTCCATGTCTCTTCGA     50
                     ||||||||||||||||||.||||.||||||||||||||||||||||||||
MbERF1_1           1 GGTCAGACTTGCCTCGCTATCTTTTCCTCATCCCTTCCATGTCTCTTCGA     50

MaERF1_1          51 CCCTCTCCGGTATATATCTTCCCCGCCCCTGCGCTTTCTCACGCTCGATA    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_1          51 CCCTCTCCGGTATATATCTTCCCCGCCCCTGCGCTTTCTCACGCTCGATA    100

MaERF1_1         101 GCGAACCCCGCGACCCAGGCGTGCATCTCATGGATCCTTCCAATCTCCAC    150
                     ||||||.||.|||.||||||||||||||||||||||||||||||||||||
MbERF1_1         101 GCGAACACCACGAACCAGGCGTGCATCTCATGGATCCTTCCAATCTCCAC    150

MaERF1_1         151 TGTCGGAGCCACGACGAGTTCTCGTCGGAATCCTCCGGCCGGTCGCCGGA    200
                     ||||||||||||||||||||||||||||||||||.|||||||||||||||
MbERF1_1         151 TGTCGGAGCCACGACGAGTTCTCGTCGGAATCCTCTGGCCGGTCGCCGGA    200

MaERF1_1         201 CTCCCTCCCCTTCAACGTCAACGACAGCGACGAGATGGTCCTGTTCGACA    250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_1         201 CTCCCTCCCCTTCAACGTCAACGACAGCGACGAGATGGTCCTGTTCGACA    250

MaERF1_1         251 TGCTGGCGGAGGCCACCGCCCCCAGGCCCGACGAGGCCAGGGACGGGGAG    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_1         251 TGCTGGCGGAGGCCACCGCCCCCAGGCCCGACGAGGCCAGGGACGGGGAG    300

MaERF1_1         301 GCCGAGTCGAAGAGCAGGGACGAGGAAGGGCTGCTGCGGCGGCGGACGCC    350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_1         301 GCCGAGTCGAAGAGCAGGGACGAGGAAGGGCTGCTGCGGCGGCGGACGCC    350

MaERF1_1         351 GGAAGATCGGTGCTACCGCGGCGTCCGGAAGCGGCCGTGGGGCAAGTTCG    400
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_1         351 GGAAGATCGGTGCTACCGCGGCGTCCGGAAGCGGCCGTGGGGCAAGTTCG    400

MaERF1_1         401 CGGCGGAGATCAGGGACTCGACCCGGAACGGGATTCGGGTGTGGTTGGGC    450
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_1         401 CGGCGGAGATCAGGGACTCGACCCGGAACGGGATTCGGGTGTGGTTGGGC    450
```

```
MaERF1_1        451 ACGTTCGACACCGCGGAGGCCGCCGCGCTGGCCTACGACCAGGCGGCGCT    500
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_1        451 ACGTTCGACACCGCGGAGGCCGCCGCGCTGGCCTACGACCAGGCGGCGCT    500

MaERF1_1        501 GTCCATGCGGGGGCAGCTCGCGGTGCTCAATTTCCCGGTGGAGCGGGTGC    550
                    |||||||||||||.||.|||||||||||||||||||||||||||||||||||
MbERF1_1        501 GTCCATGCGGGGACAACTCGCGGTGCTCAATTTCCCGGTGGAGCGGGTGC    550

MaERF1_1        551 AGGCGTCGCTGCGGGAGCTGGAGTGGGGCAAGGACGACTGCTCCCCGGTG    600
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
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MaERF1_1        601 ATGGCTCTCAAGAAGAAGCACTCCCTGAGAAGACGGCGGTCATCGAGCAT    650
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_1        601 ATGGCTCTCAAGAAGAAGCACTCCCTGAGAAGACGGCGGTCATCGAGCAT    650

MaERF1_1        651 AAAGGACAAGGTGGCGCCGACCAGGATACCGAATGTTCTGGAACTGGAAG    700
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MbERF1_1        651 AAAGGACAAGGTGGCGCCGACCAGGATACCGAATGTTCTGGAACTGGAAG    700

MaERF1_1        701 ACCTCGGCGCAGACTACTTGGAGGAGCTCCTCAGTGTATCGGAGTCTTCA    750
                    ||||.||||||||||||||||||||||||||||||||||||||.|||||
MbERF1_1        701 ACCTTGGCGCAGACTACTTGGAGGAGCTCCTCAGTGTATCGGAGCCTTCA    750

MaERF1_1        751 AAACCATGGTAACCCTTCTCCTGCTCTCCACCGCTGCCATCTCACGCCCG    800
                    ||||||||||||||||||||||||||||..||||||||||||||||||||||
MbERF1_1        751 AAACCATGGTAACCCTTCTCCTGCTCTCGGCCGCTGCCATCTCACGCCCG    800

MaERF1_1        801 GAGGACCTCATCATTTCCTCCTCCATAATTGGAGAATCCAATCACCTGCT    850
                    ||||||||||||||||||||||||||.||||||||||||||||||||||||
MbERF1_1        801 GAGGACCTCATCATTTCCTCCTCCATGATTGGAGAATCCAATCACCTGCT    850

MaERF1_1        851 CAACCTACAGCCACACTCCATGAAACTCGGATCCAGCT--CCCCCTCACC    898
                    |||||.|||||||||||||||||||||||||||||||  ||||||||||
MbERF1_1        851 CAACCCACAGCCACACTCCATGAAACTCGGATCCAGCTCCCCCCCTCACC    900

MaERF1_1        899 ATTTTTTATTCTTCTTCCCCTCTCCCTCCCTCTCTCTCTCTCTCTCTATC    948
                    ||||||||||||||||||.||||||||||||||||||||||||||||||||||
MbERF1_1        901 ATTTTTTATTCTTCTTCTCCTCTCCCTCCCTCTCTCTCTCTCTCTCTATC    950

MaERF1_1        949 GAAAGCCCCCATCAGATAAGCAGTGCTGCATTATTATGCGGGCCCCGAAA    998
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_1        951 GAAAGCCCCCATCAGATAAGCAGTGCTGCATTATTATGCGGGCCCCGAAA   1000

MaERF1_1        999 ACGATGTAAGAAAAGATGTACATGTCTGTTTCAGATCCATTGAATCCACG   1048
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_1       1001 ACGATGTAAGAAAAGATGTACATGTCTGTTTCAGATCCATTGAATCCACG   1050

MaERF1_1       1049 GGAAAGTTGAGCACGACGCCTTTGCTCTCTTCACATCA   1086
                    ||||||||||||||||||||||||||||||||||||||
MbERF1_1       1051 GGAAAGTTGAGCACGACGCCTTTGCTCTCTTCACATCA   1088
```

```
#=======================================
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# Aligned_sequences: 2
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# 2: MbERF1_2
# Matrix: EDNAFULL
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# Extend_penalty: 0.5
#
# Length: 992
# Identity:     967/992 (97.5%)
# Similarity:   967/992 (97.5%)
# Gaps:           2/992 ( 0.2%)
# Score: 4732.5
#
#
#=======================================

MaERF1_2          1 GCTCGAGAAGAAGCAAGGAGGTGGGCGAAACCCTGCGCTCTGCCTCGTTC     50
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MbERF1_2          1 GCTCGAGAAGAAGCAAGGAGGTGGGCCAAACCCTACGCTCTGCCTCGTTC     50

MaERF1_2         51 CTCCCTTTCCTCCCTATGGACTACTACCTCTTCGACTCCCTGAACCACGA    100
                    ||||||.||||||||||||||||||||||||||||||||||||||||||||
MbERF1_2         51 CTCCCTCTCCTCCCTATGGACTACTACCTCTTCGACTCCCTGAACCACGA    100

MaERF1_2        101 ACACTCGCCGGAATCCTCCACCGGTTCCACCGAGCCCTTTCCATGGGCCG    150
                    ||||||||||||||||.||.|||||||||.||||||||||.||||||||||
MbERF1_2        101 ACACTCGCCGGAATCGTCTACCGGTTCCCCCGAGCCCTTCCCATGGGCCG    150

MaERF1_2        151 GCGTTGGGCTGTTCTACCCGGACGTTCCTCTCCCTTTCAACATGGATGAC    200
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_2        151 GCGTTGGGCTGTTCTACCCGGACGTTCCTCTCCCTTTCAACATGGATGAC    200

MaERF1_2        201 TCCGAGGAGATGCTGCTGCTCGGAATGCTCGCGGAGGCCTCCGGAAAGGC    250
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MbERF1_2        201 TCCGAGGAGATGCTGCTGCTCGGAATGCTCTCGGAGGCCTCCGGAAAGGC    250

MaERF1_2        251 GTCGTCCTCGTCGGAGGCCTGCGACCGGAGCGTGATCCGGGCCAAGGAAG    300
                    ||||.||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_2        251 GTCGCCCTCGTCGGAGGCCTGCGACCGGAGCGTGATCCGGGCCAAGGAAG    300

MaERF1_2        301 AAGAGGTGGATTCGCGGAGCAAGGCGGCGGATGAGCCGAAGGAGAAGTCG    350
                    ||||.||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_2        301 AAGAAGTGGATTCGCGGAGCAAGGCGGCGGATGAGCCGAAGGAGAAGTCG    350

MaERF1_2        351 TACCGGGGGGGTGCGGAAGCGGCCGTGGGGGAAGTTCGCGGCGGAGATCAG    400
                    ||||||||||||||||||||.||||||||||||||||||||||||||||||
MbERF1_2        351 TACCGGGGGGGTGCGGAAGCGACCGTGGGGGAAGTTCGCGGCGGAGATCAG    400

MaERF1_2        401 GGACTCGACGCGGCACGGGATACGGGTGTGGCTGGGGACGTTCGACAGCG    450
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_2        401 GGACTCGACGCGGCACGGGATACGGGTGTGGCTGGGGACGTTCGACAGCG    450
```

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MaERF1_2        451 CGGAGGCCGCCGCCCTGGCGTACGACCAGGCCGCCTTCTCGATGAGGGGG    500
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_2        451 CGGAGGCCGCCGCCCTGGCGTACGACCAGGCCGCCTTCTCGATGAGGGGG    500

MaERF1_2        501 TCGATGGCGGTGCTCAATTTCCCGGTGGAGCGGGTGCGGGAGTCGTTGAA    550
                    ||||||||||||||||||||||||||||||||||||||.|||||||||||||
MbERF1_2        501 TCGATGGCGGTGCTCAATTTCCCGGTGGAGCGGGTGCAGGAGTCGTTGAA    550

MaERF1_2        551 CGGCATCAAGTGCTGGAAGGAGGAGGAGAAGGTGTCGCCGGCGGTGGCGC    600
                    ||||||||||||||||||||||||||.||.||||||||||||||||||||||
MbERF1_2        551 CGGCATCAAGTGCTGGAAGGAGGAGAAGGAGGTGTCGCCGGCGGTGGCGC    600

MaERF1_2        601 TGAAGAGGAGGCACTCCATGAGGAGGAAGTGGATGAACAAGAAAGCAAAG    650
                    ||||||||||||||||||||||||||||||||||||||.|||||||||||||
MbERF1_2        601 TGAAGAGGAGGCACTCCATGAGGAGGAAGTGGATGAGCAAGAAAGCAAAG    650

MaERF1_2        651 GAGAGTGAGACGAGCAGCAGCAGCAGCAGCAGCGTGGAGAGCGTGCTGGA    700
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_2        651 GAGAGTGAGACGAGCAGCAGCAGCAGCAGCGTGGAGAGCGTGCTGGA    700

MaERF1_2        701 GCTGGAGGACTTGGGAACAGAGTATTTGGAGGAGCTTCTGAGAACATCAG    750
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_2        701 GCTGGAGGACTTGGGAACAGAGTATTTGGAGGAGCTTCTGAGAACATCAG    750

MaERF1_2        751 AAGTAGCCAACACTTGCTGACTTCTTCCAATCCTTCTCCACCGCCAGTCT    800
                    ||||||||.|||||.|||||||||||||||||||||.|||||||||
MbERF1_2        751 AAGTAGCCGACACTAGCTGACTTCTTCCAATCCTTCTCCAACGCCAGTCT    800

MaERF1_2        801 CCCCTGTTCCTCCTTTTTTCCTAAGGGAAACCCCTCACTTGTTCCTTGTA    850
                    ||||||||||||||||||||||..||||||||||.|||||||||||||||||
MbERF1_2        801 CCCCTGTTCCTCCTTTTTTCCGGAGGGAAACCCCACACTTGTTCCTTGTA    850

MaERF1_2        851 TTCCTTTCTTGGTTTGTTCTTTCAGTTGTCCAAGTCAGGATGATCTTTTT    900
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_2        851 TTCCTTTCTTGGTTTGTTCTTTCAGTTGTCCAAGTCAGGATGATCTTTTT    900

MaERF1_2        901 TACTTGGCTGTGCTTGGCATG--TGCCATACCAAGATATCTCGATATCTT    948
                    ||||||||||||||||||||||  |||||.||||||||||||.||||||||
MbERF1_2        901 TACTTGGCTGTGCTTGGCATGGATGCCACACCAAGATATCTTGATATCTT    950

MaERF1_2        949 ATTTCCCTGCTGCAAATCAATATAGCTTTTGATCCTGTAAAA    990
                    ||||||||||||||||||||||||||||||||||||||||||||
MbERF1_2        951 ATTTCCCTGCTGCAAATCAATATAGCTTTTGATCCTGTAAAA    992
```

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#=======================================
#
# Aligned_sequences: 2
# 1: MaERF1_3
# 2: MbERF1_3
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 808
# Identity:     793/808 (98.1%)
# Similarity:   793/808 (98.1%)
# Gaps:           2/808 ( 0.2%)
# Score: 3893.0
#
#
#=======================================

MaERF1_3           1 GCCTGAGAACCACCGATCTCCCACCCCACAATTCACGATGGATCCTTCAT     50
                     ||||.||||||||||||||||||||||||||||||||||||||||||||
MbERF1_3           1 GCCTAAGAACCACCGATCTCCCACCCCACAATTCACGATGGATCCTTCAT     50

MaERF1_3          51 ATCTCCAGTCCCAGAGTTACGACGAATTCTCGCCGGAAGATTCCTATCGC    100
                     ||||.||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_3          51 ATCTGCAGTCCCAGAGTTACGACGAATTCTCGCCGGAAGATTCCTATCGC    100

MaERF1_3         101 CTCCCCTTCGACGTCAACGACAGCGACGAGATGCTCCTGTTCGACACACT    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_3         101 CTCCCCTTCGACGTCAACGACAGCGACGAGATGCTCCTGTTCGACACACT    150

MaERF1_3         151 GGCGGAGGCCACCCCTTCGAACCCGGTCCTGGCAGGGGAGGGTCGACCGA    200
                     ||||||||||||||||||.|||||||||||||||||||||.|||||||
MbERF1_3         151 GGCGGAGGCCACCCCTTCTAACCCGGTCCTGGCAGGGGAGGGCCGACCGA    200

MaERF1_3         201 CGGGCGAGCCGTGCTACCGCGGCGTCCGTAAGCGGCCGTGGGGGAAGTTC    250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_3         201 CGGGCGAGCCGTGCTACCGCGGCGTCCGTAAGCGGCCGTGGGGGAAGTTC    250

MaERF1_3         251 GCGGCGGAGATAAGGGACTCGACGCGGGGAGGGGCGCGGGTGTGGCTGGG    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_3         251 GCGGCGGAGATAAGGGACTCGACGCGGGGAGGGGCGCGGGTGTGGCTGGG    300

MaERF1_3         301 GACGTTCGACACCGCGGAGGCCGCCGCCCTGGCGTACGACCAGGCGGCGT    350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_3         301 GACGTTCGACACCGCGGAGGCCGCCGCCCTGGCGTACGACCAGGCGGCGT    350

MaERF1_3         351 TCTCCATGCGGGGGCGGCTCGCCGTGCTCAACTTCCCAGTGGAGCAGGTG    400
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_3         351 TCTCCATGCGGGGGCGGCTCGCCGTGCTCAACTTCCCAGTGGAGCAGGTG    400

MaERF1_3         401 CAGGAGTCCTTGCAAGAGCTCGAATGGGATAAGGACAACTGCTCCCCCAT    450
                     |||||||||||||||.|||||||||||||||||||.|||||||||||||||
MbERF1_3         401 CAGGAGTCCTTGCAGGAGCTCGAATGGGATAAGGATAACTGCTCCCCCAT    450
```

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MaERF1_3        451 CATGGCACTCAAGAAGAAGCACTCGTTAAGGAGGAGGAGGAGACCTGCAG  500
                    ||||||||||||||||||||||||||.||||||||||||||||||||||||
MbERF1_3        451 CATGGCACTCAAGAAGAAGCACTCATTAAGGAGGAGGAGGAGACCTGCAG  500

MaERF1_3        501 TGAGCGGGAAGACCAAGGTGGCACAGAGCAGGATACAGAGTGTCCTGGAA  550
                    ||||||||||||||||||||||||||||||||||||.|||||||||||||.|||
MbERF1_3        501 TGAGCGGGAAGACCAAGGTGGCACAGAGCAGGAGACAGAGTGTCCTAGAA  550

MaERF1_3        551 CTGGAGGACTTGGGCACAGATTACTTGGAGGAGTTACTGAGAGTTTCCGA  600
                    |||||||||||||||.||||||||||||||||||||||||||||||||||||
MbERF1_3        551 CTGGAGGACTTGGGAACAGATTACTTGGAGGAGTTACTGAGAGTTTCCGA  600

MaERF1_3        601 ACTTGCATAA-CTCAGTAAACCTGCTCCCTGCAGCTCAAATCAAACTCCA  649
                    |||||||||| |||||||||||||||||||||||.|||||||||||||||
MbERF1_3        601 ACTTGCATAACCTCAGTAAACCTGCTCCCTGCAGCTAAAATCAAACTCCA  650

MaERF1_3        650 TGGAACTCGGATCCAGCTTTCGGTTCCTTCATCATTATTTATTCTGCTTG  699
                    ||||||||||||||||||||||||.||||||||||||||||||||||||||
MbERF1_3        651 TGGAACTCGGATCCAGCTTTCGATTCCTTCATCATTATTTATTCTGCTTG  700

MaERF1_3        700 CATCATTACTTGGTCCCCCAAAATGATGTAACAGGAAAATGTATGTGTTT  749
                    ||||||||||||||||||||||||.||||||||||||||||||||||||||
MbERF1_3        701 CATCATTACTTGGTCCCCCAAAACGATGTAACAGGAAAATGTATGTGTTT  750

MaERF1_3        750 CAGATCCGTTGAATCCATGC-AAAGATGAGCGCGATGGCTTTGCTTGCTT  798
                    |||||||||||||||||||| ||||||||||||||||||||||||||||||
MbERF1_3        751 CAGATCCGTTGAATCCATGCAAAAGATGAGCGCGATGGCTTTGCTTGCTT  800

MaERF1_3        799 TACACCAA    806
                    ||||||||
MbERF1_3        801 TACACCAA    808
```

```
#=======================================
#
# Aligned_sequences: 2
# 1: MaERF1_4
# 2: MbERF1_4
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 1054
# Identity:    1032/1054 (97.9%)
# Similarity:  1032/1054 (97.9%)
# Gaps:           1/1054 ( 0.1%)
# Score: 5066.0
#
#
#=======================================

MaERF1_4          1 TGAGGCTCTTATGCCGCGAAACCACACGAGCCGAGCAAGAAGAAGCCTGT     50
                    |||||||||.||||||||||||||||.|.||||||||||||||||||||
MbERF1_4          1 TGAGGCTCTAATGCCGCGAAACCACAAGGGCCGAGCAAGAAGAAGCCTGT     50

MaERF1_4         51 TTCTCCGTCGATGGATTCTCCAAACCTCTACTTCCGCTGCTCCGAATCCT    100
                    ||||||||||||||||||||||||||||||||||.|||.|||||||||||||
MbERF1_4         51 TTCTCCGTCGATGGATTCTCCAAACCTCTACTTGCGCAGCTCCGAATCCT    100

MaERF1_4        101 CGGCCGCGTCCACACCCGAATCCCCGGAACCTGCCCCGTGCTCCCGCCTC    150
                    ||||||||||||||||||||||||||.|.||||.||||||||||||||||
MbERF1_4        101 CGGCCGCGTCCACACCCGAATCCCCGAAGCCTGTCCCGTGCTCCCGCCTC    150

MaERF1_4        151 GACCAACCGCTTCCCTTCGACGTGAACGACGCCGATGAGATGCTCTTGCT    200
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_4        151 GACCAACCGCTTCCCTTCGACGTGAACGACGCCGATGAGATGCTCTTGCT    200

MaERF1_4        201 GGACATGCTCATCGATGCTCCCGACGTGTCTAACTCTACCATGGCGGCAG    250
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_4        201 GGACATGCTCATCGATGCTCCCGACGTGTCTAACTCTACCATGGCGGCAG    250

MaERF1_4        251 AAGAGGTCGGGTCGAGCGTGACGGCGGAGCCCCCGGGGAGCGAGAAGAGC    300
                    ||||||||||||||||||||||||||||||||.|||||.||||||||||||
MbERF1_4        251 AAGAGGTCGGGTCGAGCGTGACGGCGGAGCCCTCGGGGGGCGAGAAGAGC    300

MaERF1_4        301 TACAGAGGGGTGCGGAAGCGGCCGTGGGGGAAGTTCGCGGCGGAGATCAG    350
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_4        301 TACAGAGGGGTGCGGAAGCGGCCGTGGGGGAAGTTCGCGGCGGAGATCAG    350

MaERF1_4        351 GGACTCGACGCGGCAGGGGGTGAGAGTGTGGCTGGGCACGTTCGACGACG    400
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_4        351 GGACTCGACGCGGCAGGGGGTGAGAGTGTGGCTGGGCACGTTCGACGACG    400

MaERF1_4        401 CGGAGGCGGCCGCCTTGGCCTACGACCAGGCGGCATTGGCGATGAGGGGG    450
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_4        401 CGGAGGCGGCCGCCTTGGCCTACGACCAGGCGGCATTGGCGATGAGGGGG    450
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MaERF1_4        451 ACGGCGGCGGTGCTCAATTTCCCGGCCGAGCGTGTGCGGGCGTCGCTGCG    500
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_4        451 ACGGCGGCGGTGCTCAATTTCCCGGCCGAGCGTGTGCGGGCGTCGCTGCG    500

MaERF1_4        501 GGACCTCGAGCTGGGGGTGGATGGGTGTTCCCCGGTTCTGGCACTGAAGA    550
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_4        501 GGACCTCGAGCTGGGGGTGGATGGGTGTTCCCCGGTTCTGGCACTGAAGA    550

MaERF1_4        551 AGAGGCACTGCATCAGGAAGAGGAGGAGGTCAGGGGGCAAGGTAATGGAG    600
                    ||||||||||||||||||||||||||||||||||||||||||.||.||||
MbERF1_4        551 AGAGGCACTGCATCAGGAAGAGGAGGAGGTCAGGGGGCAAGGAAAGGGAG    600

MaERF1_4        601 AGTGTTGTGGTCTTGGAGGACTTGGGAGCAGAGTACTTGGAGGAACTCTT    650
                    ||.|||||||||||||||||||||||||||||||.|||||||||.|||||
MbERF1_4        601 AGAGTTGTGGTCTTGGAGGACTTGGGAGCAGAGTATTTGGAGGAGCTCTT    650

MaERF1_4        651 GAGACTGTCAGAACCTGCAAGTCCTTGGTGATCATCCAATGGTTTTGCTT    700
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_4        651 GAGACTGTCAGAACCTGCAAGTCCTTGGTGATCATCCAATGGTTTTGCTT    700

MaERF1_4        701 TCCTGTAGCTTGTTCGTTGTGATGACAAACAGCTTCACAAGTCTAATGAT    750
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_4        701 TCCTGTAGCTTGTTCGTTGTGATGACAAACAGCTTCACAAGTCTAATGAT    750

MaERF1_4        751 TTGCTCATTCCCATAAAATCTGGATCCATCTTCTTTCTTGATGCTTCACT    800
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_4        751 TTGCTCATTCCCATAAAATCTGGATCCATCTTCTTTCTTGATGCTTCACT    800

MaERF1_4        801 GTATATTAAACCATGGCTGCATCATCATGTGGCCTACACAACGAGCAAAG    850
                    |||||.|||||||||||||||||||||||||||.|||||||||||||||||
MbERF1_4        801 GTATAGTAAACCATGGCTGCATCATCATGTGGCCTTCACAACGAGCAAAG    850

MaERF1_4        851 ATCT-TCTCTCTCTCTCTCTCAATTTAAGATACATGCTCAGATTACCA    899
                    |||| ||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_4        851 ATCTCTCTCTCTCTCTCTCTCAATTTAAGATACATGCTCAGATTACCA    900

MaERF1_4        900 TGTCAAAAGGTAGGCTATGCCAAAAGAGGATGTCATGTTGCCTTGTTTCA    949
                    |.|||||||||||||||||||||||||||||||||||||||||.|||||
MbERF1_4        901 TTTCAAAAGGTAGGCTATGCCAAAAGAGGATGTCATGTTGCCTTTTTTCA    950

MaERF1_4        950 ATGAGCTCCATGTATTGCTAGACTCTGTCCAACTGAGTTTACGGCTCATA    999
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_4        951 ATGAGCTCCATGTATTGCTAGACTCTGTCCAACTGAGTTTACGGCTCATA   1000

MaERF1_4       1000 TTGTTTGGATTGGAAGAGTTGATCTGAAACCATCTGAGATATGGGAGTGA   1049
                    ||.|||||||||||||||||||||||||||||||||||.||||||||||||
MbERF1_4       1001 TTATTTGGATTGGAAGAGTTGATCTGAAACCATCTGAGGTATGGGAGTGA   1050

MaERF1_4       1050 GACA   1053
                    ||||
MbERF1_4       1051 GACA   1054
```

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#=======================================
#
# Aligned_sequences: 2
# 1: MaERF1_5
# 2: MbERF1_5
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 706
# Identity:     685/706 (97.0%)
# Similarity:   685/706 (97.0%)
# Gaps:           6/706 ( 0.8%)
# Score: 3324.0
#
#
#=======================================

MaERF1_5          1 GACCTCCTTCCAAGGTTCCGAATCCTCATCGATATCCACAGCCGGATCGC     50
                    ||||||||||||||||||||||||||||||||.|.||||||||||||||||
MbERF1_5          1 GACCTCCTTCCAAGGTTCCGAATCCTCATCGGTGTCCACAGCCGGATCGC     50

MaERF1_5         51 AGGAGGAATCTCTCCCCTTCGACGTGAACGACGCCGGTGAGATGCTCCTG    100
                    |||||||.||||||||||||||||||||||||||||||||||||||||||||
MbERF1_5         51 AGGAGGAGTCTCTCCCCTTCGACGTGAACGACGCCGGTGAGATGCTCCTG    100

MaERF1_5        101 TTCGACATGCTCATCGAGTCCGCCATGACCACGAAGACGTCGACGGGCAA    150
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_5        101 TTCGACATGCTCATCGAGTCCGCCATGACCACGAAGACGTCGACGGGCAA    150

MaERF1_5        151 AGAGGCGGAGTCGAAGGGCCCGACGGCGAGCGGGAAGAGCTACCGAGGGG    200
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_5        151 AGAGGCGGAGTCGAAGGGCCCGACGGCGAGCGGGAAGAGCTACCGAGGGG    200

MaERF1_5        201 TGCGGAGGCGGCCGTGGGGCAAGTTCGCGGCTGAGATCAGGGACTCGACG    250
                    ||||||||||||||||||||||||||||||||.||||||||||||||||||
MbERF1_5        201 TGCGGAGGCGGCCGTGGGGCAAGTTCGCGGCGGAGATCAGGGACTCGACG    250

MaERF1_5        251 CGGCAGGGGGTGCGGGTGTGGCTGGGCACGTTCGACAGCGCGGAGGCCGC    300
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_5        251 CGGCAGGGGGTGCGGGTGTGGCTGGGCACGTTCGACAGCGCGGAGGCCGC    300

MaERF1_5        301 CGCCCTGGCCTACGACCAGGCGGCGCTGTCGATGAGGGGGGCGACGGCGG    350
                    ||||||||||||||||||||||||||||||||||||||||||||||||||.|
MbERF1_5        301 CGCCCTGGCCTACGACCAGGCGGCGCTGTCGATGAGGGGGGCGACGGCCG    350

MaERF1_5        351 TGCTCAACTTTCCGGCGGAGCGCGTGCGGGAGTCGCTGCGGGGGCTGGAG    400
                    ||||||||||.||||||||||||||||||||||.||||||||||||||||||
MbERF1_5        351 TGCTCAACTTCCCGGCGGAGCGCGTGCGGGAGTCGCTGCAGGGGCTGGAG    400

MaERF1_5        401 CTGGCGAAGGACGGGTGCTCCCCGGTGGTGGCGCTGAAGAAGAAGCACTG    450
                    ||||||||||||||.||||||||||||||||||||||||||||||||||||
MbERF1_5        401 CTGGCGAAGGACGGCTGCTCCCCGGTGGTGGCGCTGAAGAAGAAGCACTG    450
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MaERF1_5        451 CATGAGGAGGAGGAGGAAGAGAAAGGTGAGGGAGTCGAGTGGGGAGGAGG    500
                    |||.|||||||||||||||||||||||||||||||||||||||   |||||
MbERF1_5        451 CATCAGGAGGAGGAGGAAGAGAAAGGTGAGGGAGTCGAGTGG---GGAGG    497

MaERF1_5        501 GCGTAGTGGAATTAGAGGA-CTTGGGAGTGGAGTTCTTGGAGGACCTCTT    549
                    |||||||||||||||||| .|||||||||||||||||||||||||||||
MbERF1_5        498 GCGTAGTGGAATTAGAGGATTTTGGGAGTGGAGTTCTTGGAGGACCTCTT    547

MaERF1_5        550 GGGGCTTTCAGGGCTTGCGAGTCAGTGATGACAAGCTCATAGTTTTGCCC    599
                    ||||||.|||||||||||||||||||.|||||||||||||||||||||||
MbERF1_5        548 GGGGCTATCAGGGCTTGCGAGTCAGTGGTGACAAGCTCATAGTTTTGCCC    597

MaERF1_5        600 AACCTAATGATATTTTAAATATATTAATATGGATATTAAGTTGACTGTCA    649
                    |||||.|||||||||||||||||||||||||||||||||||||||||||||
MbERF1_5        598 AACCTTATGATATTTTAAATATATTAATATGGATATTAAGTTGACTGTCA    647

MaERF1_5        650 ATTAGATTTACTGTAATGGACACATGTGCAAGT-TTTTGTATAGTTTTGT    698
                    |||||||||||||||| |||||||||||||| ||||.|||||||.|||
MbERF1_5        648 ATTAGATTTACTGTAAT-GACACATGTGCAAGTGTTTTTTATAGTTATGT    696

MaERF1_5        699 TAAGAT    704
                    ||||||
MbERF1_5        697 TAAGAT    702
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#=======================================
#
# Aligned_sequences: 2
# 1: MaERF1_6
# 2: MbERF1_6
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 927
# Identity:     899/927 (97.0%)
# Similarity:   899/927 (97.0%)
# Gaps:           8/927 ( 0.9%)
# Score: 4401.5
#
#
#=======================================

MaERF1_6          1 AGCCGTCCTTACGATCAAGCTTAGCAGTTGCACTGATACTACTGAGACAA     50
                    ||||||||||.|||||||||||.|||.||||||||||||||||||||
MbERF1_6          1 AGCCGTCCTTATGATCAAGCTTAGTAGTAGCACTGATACTACTGAGACAA     50

MaERF1_6         51 GCGGAAGGCAGAAGCAATCTCTGGCTTGTCTTCCTCTTCGATGGATTACT    100
                    |.||||||||||||||||||..||..||||||||||||.||||||||||
MbERF1_6         51 GAGGAAGGCAGAAGCAATCCCTATCTTGTCTTCCTCTTCAATGGATTACT    100

MaERF1_6        101 CTCTCTCCTTTCACTCCCATAACCAGGAACACTCATCTGAGTCCTCCACG    150
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_6        101 CTCTCTCCTTTCACTCCCATAACCAGGAACACTCATCTGAGTCCTCCACG    150

MaERF1_6        151 TACTCGCCCAGGTCCTCGGCAACCGACGGCTTCGGGCTCGTCTGCCCTGA    200
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_6        151 TACTCGCCCAGGTCCTCGGCAACCGACGGCTTCGGGCTCGTCTGCCCTGA    200

MaERF1_6        201 CAAGCCCCTTCCGTTCGACGAGAACGACTCCGAGGAGATGCTGCTGCTTA    250
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_6        201 CAAGCCCCTTCCGTTCGACGAGAACGACTCCGAGGAGATGCTGCTGCTTA    250

MaERF1_6        251 GCATGCTCGCAGAGGCCTCAGGCAAGGCGGCGTCGTCGTCCTCGTCGGAG    300
                    |||||||||||||||||||||||||||||.||||||||||||||||||||
MbERF1_6        251 GCATGCTCGCAGAGGCCTCAGGCAAGGCGTCGTCGTCGTCCTCGTCGGAG    300

MaERF1_6        301 GTCCTTGACAGCCGCAGTTCACCCCGACCCAAGGAAGAAGAGGTGGAATC    350
                    |||||||        |.||.||||||||||||||||||||||||||||
MbERF1_6        301 GTCCTTG--------ATTTTACCCCGACCCAAGGAAGAAGAGGTGGAATC    342

MaERF1_6        351 GAGAAGCAAGGTGGGTCATGACACAAAGGGAGAGAAGCCCTACCGCGGGG    400
                    |||||||||||||||||||||||||||||||||||||.||||||||||||
MbERF1_6        343 GAGAAGCAAGGTGGGTCATGACACAAAGGGAGAGAAGTCCTACCGCGGGG    392

MaERF1_6        401 TGAGACGGCGGCCGTGGGGGAAGTTCGCCGCCGAGATAAGAGACTCAACG    450
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_6        393 TGAGACGGCGGCCGTGGGGGAAGTTCGCCGCCGAGATAAGAGACTCAACG    442
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MaERF1_6        451 CGGCGCGGGATTCGCGTGTGGCTGGGAACGTTCGACAGCGCGGAGGCAGC    500
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_6        443 CGGCGCGGGATTCGCGTGTGGCTGGGAACGTTCGACAGCGCGGAGGCAGC    492

MaERF1_6        501 TGCGCTGGCTTACGACCAGGCGGCGTTCTCGATGCGGGGGACGACGGCGG    550
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_6        493 TGCGCTGGCTTACGACCAGGCGGCGTTCTCGATGCGGGGGACGACGGCGG    542

MaERF1_6        551 TGCTCAATTTCCCGGTGGAGAGAGTTCGGGAGTCGCTGCGGGGCGTGAAG    600
                    |.||||||||||||||||.||||||||||||||||||||||||||||||||
MbERF1_6        543 TCCTCAATTTCCCGGTGGACAGAGTTCGGGAGTCGCTGCGGGGCGTGAAG    592

MaERF1_6        601 TACGAGGAGGAGGAGATTGGGCTGTCGCCCGTGGTGGCGCTCAAGCGGAG    650
                    ||||.||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_6        593 TACGCGGAGGAGGAGATTGGGCTGTCGCCCGTGGTGGCGCTCAAGCGGAG    642

MaERF1_6        651 GAATACCCTGAGGAGGAAGTCGACGAGCAAGAAGGCCAAAGGCCGGGAGG    700
                    ||||||||||||||||||||||||||||||||||||||||||..||||||
MbERF1_6        643 GAATACCCTGAGGAGGAAGTCGACGAGCAAGAAGGCCAAAGGGAGGGAGG    692

MaERF1_6        701 TGAGGACGGCGGAGAGTGTGGTGGAGTTGGAGGACCTGGGAGCAGAGTAC    750
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_6        693 TGAGGACGGCGGAGAGTGTGGTGGAGTTGGAGGACCTGGGAGCAGAGTAC    742

MaERF1_6        751 TTGGAGGAGCTCTTGAGCACCTCAGGGTTTGCCAGGCCGTGGTGAACCGC    800
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_6        743 TTGGAGGAGCTCTTGAGCACCTCAGGGTTTGCCAGGCCGTGGTGAACCGC    792

MaERF1_6        801 AACTCTCAATCCTCGAGACCATGTTCTCTGTATACCTTTCTTGTTTCCTT    850
                    |||||||||||||.|||||||.||||||||||||||||||||||||||||||
MbERF1_6        793 AACTCTCAATCCTCAAGACCACGTTCTCTGTATACCTTTCTTGTTTCCTT    842

MaERF1_6        851 TCTTCTTTCCTTCGTTCAATTGTTCCAATCCTGCAGCACAAAGAAGCTCT    900
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_6        843 TCTTCTTTCCTTCGTTCAATTGTTCCAATCCTGCAGCACAAAGAAGCTCT    892

MaERF1_6        901 AAGAATTCTACTTCTTTCTCTGTTCCA    927
                    ||||||||||||||||.||||||||||
MbERF1_6        893 AAGAATTCTACTTCTTCCTCTGTTCCA    919
```

```
#=======================================
#
# Aligned_sequences: 2
# 1: MaERF1_7
# 2: MbERF1_7
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 1417
# Identity:    1379/1417 (97.3%)
# Similarity:  1379/1417 (97.3%)
# Gaps:           3/1417 ( 0.2%)
# Score: 6725.0
#
#
#=======================================

MaERF1_7          1 ACTTGCAAGGGGTGAAATCCCAATAACTCAAACAAAAATGCTTTTGATTA     50
                    ||||||||||||||||||||||.||.||||||||||||||||||||||||
MbERF1_7          1 ACTTGCAAGGGGTGAAATCCCGATTACTCAAACAAAAATGCTTTTGATTA     50

MaERF1_7         51 ATTCTCCTTGATGATAGAAGCCATACCTTGCCTTCCTCAGCTCTTTCACT    100
                    ||||||||||||||||||||||||.||||||||||||||||||||||||||
MbERF1_7         51 ATTCTCCTTGATGATAGAAGCCATGCCTTGCCTTCCTCAGCTCTTTCACT    100

MaERF1_7        101 TTCACACTAGAGTTTGCACAGGTCACCAATGCAGCACTCTAACCCAGCTC    150
                    |||||||||||||||||||||||||||||||||||.|||||||||||||||
MbERF1_7        101 TTCACACTAGAGTTTGCACAGGTCACCAATGCAGCATTCTAACCCAGCTC    150

MaERF1_7        151 TGAGAGAGAGAGAGAGAGAGAGAGAGAGAGCGTGTGTGTGTGGTGGCTGC    200
                    |.|||||||||||||||||||||||||||.|.|||.|||||||||||||||
MbERF1_7        151 TCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGTGAGTGTGGTGGCTGC    200

MaERF1_7        201 AAATACAAGAGGACAGCACAAGAGATCTCCCACCAAAAACTGTCACCCTG    250
                    |||||||||||||||||||||||||||||||||||||||||||||||| ||.|
MbERF1_7        201 AAATACAAGAGGACAGCACAAGAGATCTCCCACCAAAAACTGTCA-CCAG    249

MaERF1_7        251 CAGCGATGGCTTCAACACGGGACATGGAGCCACGAAGTGGCCACCAATGA    300
                    ||||||| |||||||||||||||||||||||.||||||||||.|||
MbERF1_7        250 CAGCGAT-GCTTCAACACGGGACATGGAGCCACGACGTGGCCACCACTGA    298

MaERF1_7        301 TGTCACCGTGCTCGTCTCACGGATACTTCACCCGACCTCTGCCGCCGTGC    350
                    |||||||||||||||||||||||||||||||||||||||||||||||||||.
MbERF1_7        299 TGTCACCGTGCTCGTCTCACGGATACTTCACCCGACCTCTGCCGCCGTGA    348

MaERF1_7        351 GTGGCTGACCCACGTGCTCCTTTGGCCCGGCACGAACCTTTTCCCGCCAC    400
                    |||||||||.|||||||||||||||||||||||||||||||||||.|||
MbERF1_7        349 GTGGCTGACTCACGTGCTCCTTTGGCCCGGCACGAACCTTTTCCCGTCAC    398

MaERF1_7        401 CCGCAACTCCCAGGTGGCCCCAGTCAATCTATCCATCGATTCCCCCTCTC    450
                    |||||||||||||||||||||||||||||||||||.|||||||||||||||
MbERF1_7        399 CCGCAACTCCCAGGTGGCCCCAGTCAATCTATCCATTGATTCCCCCTCTC    448
```

```
MaERF1_7        451 TCGTTCAAGTATAAGAAGGAGCGGGCTTAGATCCTGTCGACTGGACGAGC    500
                    ||||||||||||||||||||||||||||||||||||||||||||||.|||
MbERF1_7        449 TCGTTCAAGTATAAGAAGGAGCGGGCTTAGATCCTGTCGACTGGACAAGC    498

MaERF1_7        501 TCGAGCACGAACAAGGAGGTGGGAGACACCCTGATTTCTCTCTCTCCTCA    550
                    ||||||||||||||||.||||||||||||||||||||||.||||||
MbERF1_7        499 TCGAGCACGAACAAGGCGGTGGGAGACACCCTGATTTCTCTCTTTCCTCA    548

MaERF1_7        551 TCCCTTCCTTTCTCCCCCCCATGGACTACTCCCTCTTCCAGTCGCTACAC    600
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_7        549 TCCCTTCCTTTCTCCCCCCCATGGACTACTCCCTCTTCCAGTCGCTACAC    598

MaERF1_7        601 TCGCCGGAATCTTCCACTGGCTCCGGCGACCCCTTCCCCTGGACCGGCGT    650
                    ||||||||||||||||||||||||||||.||||||||||||||||||||||
MbERF1_7        599 TCGCCGGAATCTTCCACTGGCTCCGGCAACCCCTTCCCCTGGACCGGCGT    648

MaERF1_7        651 CGGGCTGTTCTACCCGGACGTTCCTGTCCCGTTCGACATGAACGACTCCG    700
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_7        649 CGGGCTGTTCTACCCGGACGTTCCTGTCCCGTTCGACATGAACGACTCCG    698

MaERF1_7        701 AGGAGATGCTCCTCCTCGGAATGCTCGCGGAGGCCTCCGGTAAGGCGTCG    750
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_7        699 AGGAGATGCTCCTCCTCGGAATGCTCGCGGAGGCCTCCGGTAAGGCGTCG    748

MaERF1_7        751 TCCTCGTTAGAGGCCTGCGAGCGCAGCCCAGCCCAGCCCAAGGAGGAAGA    800
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_7        749 TCCTCGTTAGAGGCCTGCGAGCGCAGCCCAGCCCAGCCCAAGGAGGAAGA    798

MaERF1_7        801 GGTGGATTCGCAGAGCAAGGTGGCGGACGATCCCAAGGTGAAGTCGTACC    850
                    ||||||||||.||||||||||||||||||||||.||||||||||||
MbERF1_7        799 GGTGGATTCGCGGAGCAAGGTGGCGGACGATCCCAAGGAGAAGTCGTACC    848

MaERF1_7        851 GGGGGGTGAGAAAGCGGCCGTGGGGGAAGTTCGCGGCGGAGATCCGGGAC    900
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_7        849 GGGGGGTGAGAAAGCGGCCGTGGGGGAAGTTCGCGGCGGAGATCCGGGAC    898

MaERF1_7        901 TCGACGCGGCACGGCATACGGGTGTGGCTGGGAACGTTCGACAGCGCGGA    950
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_7        899 TCGACGCGGCACGGCATACGGGTGTGGCTGGGAACGTTCGACAGCGCGGA    948

MaERF1_7        951 GGCCGCGGCGCTGGCGTACGACCAGGCCGCCTTCTCGATGCGGGGCTCGA   1000
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MbERF1_7        949 GGCCGCGGCGCTGGCGTACGACCAGGCCGCCTTCTCGATGCGGGGCTCGA    998

MaERF1_7       1001 CGGCGGTGCTCAATTTCCCGGTGGACCGGGTGCGGGAGTCGCTGAACGGC   1050
                    ||||||||||||||||||||||||||||||||||||||||.|||||||||
MbERF1_7        999 CGGCGGTGCTCAATTTCCCGGTGGACCGGGTGCGGGAGTCACTGAACGGC   1048

MaERF1_7       1051 ATGAAATGCTGGGATGAACAGGAGGAGGAGGGGGTGTCGCCGGTGGTGGT   1100
                    ||||||||||||||.||||||||||||.||||||||||||||||||||||
MbERF1_7       1049 ATGAAATGCTGGGAGGAACAGGAGGAGAAGGGGGTGTCGCCGGTGGTGGT   1098
```

```
MaERF1_7        1101 GCTGAAGAGGAAGCACTCCATGAGGAGGAAGTCGATGGGCAAGAAGGCAA   1150
                     ||||||||||||||||||||||||||||||||||||..||||||||||||
MbERF1_7        1099 GCTGAAGAGGAAGCACTCCATGAGGAGGAAGTCGATGACCAAGAAGGCAA   1148

MaERF1_7        1151 AGCAGAGCGAGACGAGCATTCGTAGCGCGGAGAGCGTGTTGGAGCTAGAG   1200
                     |||||||||||||.||||||||||||||||||||||||||||||||||||
MbERF1_7        1149 AGCAGAGCGAGACAAGCATTCGTAGCGCGGAGAGCGTGTTGGAGCTAGAG   1198

MaERF1_7        1201 GACTTAGGAGCAGAGTACTTGGAACAGCTTCTCACAACATCAGAGGTTGC   1250
                     ||||||||||||||||||||||||||||||||||.||||||||||||||.
MbERF1_7        1199 GACTTAGGAGCAGAGTACTTGGAACAGCTTCTCACTACATCAGAGGTTGT   1248

MaERF1_7        1251 GCCAATCCATGCTCAGTGTTAAGCATCTGTCTTGTGTTTTTTCTGTGGGA   1300
                     ||||||||||||.||||||||||||||||||.||||||||||||||||||
MbERF1_7        1249 GCCAATCCATGCCCAGTGTTAAGCATCTGTCATGTGTTTTTTCTGTGGGA   1298

MaERF1_7        1301 AATCTCTCCCCTGTTCATCAAATTTGTTTCTTGTTTTGTTCTTCCAGTTG   1350
                     |||||||.||||||||||||||||.||||||||||||||||||||||||||
MbERF1_7        1299 AATCTCTTCCCTGTTCATCAAATTTCTTTCTTGTTTTGTTCTTCCAGTTG   1348

MaERF1_7        1351 TCCAAG-TAATAAGAACCTCCTTTTTACTTGGCTACGTTTGTGATAGTAA   1399
                     |||||| ||..||||||||||||||||||||||||||||||||||||||||
MbERF1_7        1349 TCCAAGTTACAAAGAACCTCCTTTTTACTTGGCTACGTTTGTGATAGTAA   1398

MaERF1_7        1400 GAGATTTTGAGATCTTA   1416
                     |||||||||||||||||
MbERF1_7        1399 GAGATTTTGAGATCTTA   1415
```

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(REGULAR ISSUE)

(Manuscript Preparation & Submission Guide)

*Revised: December 2020*

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- that have not been published elsewhere (including proceedings)
- that are not currently being submitted to other journals

#### 1. Regular article

Regular article is a full-length original empirical investigation, consisting of introduction, methods, results, and discussion. Original research work should present new and significant findings that contribute to the advancement of the research area. *Analysis and Discussion* must be supported with relevant references.

*Size:* Generally, each manuscript is **not to exceed 6000 words** (excluding the abstract, references, tables, and/or figures)**,** a maximum of **80 references**, and **an abstract of less than 250 words.**

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A review article reports a critical evaluation of materials about current research that has already been published by organising, integrating, and evaluating previously published materials. It summarises the status of knowledge and outlines future directions of research within the journal scope. A review article should aim to provide systemic overviews, evaluations, and interpretations of research in a given field. Re-analyses as meta-analysis and systemic reviews are encouraged.

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Each article should be timely and brief. It is suitable for the publication of significant technical advances and maybe used to:

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**Tables/figures list:** A **list** of the number of **black and white/colour figures and tables** should also be indicated on this page. See ***"5. Figures & Photographs"*** for details.

*Example (page 2):*

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**Nur Atheeqah-Hamzah, Christina Seok Yien Yong* and Umi Kalsom Yusuf**

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*Table: 1*
*Figure 1.*

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This page should **repeat** the **full title** of your paper with only the ***Abstract***, usually in one paragraph and ***Keywords.***

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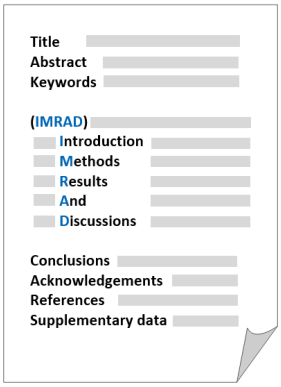


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  Example:

  Table 1
  *PVY infected* Nicotiana tabacum *plants optical density in ELISA*
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- Each table should be prepared on a new page, embedded in the manuscript.
- Authors are advised to keep backup files of all tables.

***** Please submit all tables in Microsoft word format only, because tables submitted as image data cannot be edited for publication and are usually in low-resolution.***

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