Pertanika Journal of

# TROPICAL AGRICULTURAL SCIENCE

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Journal of Tropical Agricultural Science

# *Journal of Tropical Agricultural Science*

## About the Journal

### Overview

Pertanika Journal of Tropical Agricultural Science (JTAS) is the official journal of Universiti Putra Malaysia published by UPM Press. It is an open-access online scientific journal which is free of charge. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognized 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.

JTAS 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 to authors around the world regardless of the nationality.

The Journal is available world-wide.

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

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

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## International Standard Serial Number (ISSN)

An ISSN is an 8-digit code used to identify periodicals such as journals of all kinds and on all media–print and electronic. All Pertanika journals have ISSN as well as an e-ISSN.

Journal of Science & Technology: ISSN 0128-7680 (*Print*); ISSN 2231-8526 (*Online*).

## Lag time

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In the peer-review process, three referees independently evaluate the scientific quality of the submitted manuscripts.

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.

## Operating and review process

What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are seven steps to the editorial review process:

1. The Journal's Chief Executive Editor (CEE) and the Editorial Board Members (EBMs) examine the paper to determine whether it is appropriate for the journal and should be reviewed. If not appropriate, the manuscript is rejected outright and the author is informed.

2. The CEE sends the article-identifying information having been removed, to three reviewers who are specialists in the subject matter represented by the article. The CEE requests them to complete the review in three 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 field.

3. The CEE, in consultation with the Editor-in-Chief (EiC), examines the reviews and decides whether to reject the manuscript, invites the author(s) to revise and resubmit the manuscript. The CEE may seek additional reviews. Final acceptance or rejection rests with the CEE and EiC, who reserve the right to refuse any material for publication. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the author) are forwarded to the author. If a revision is indicated, the editor provides guidelines 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 author(s) may also submit a rebuttal if there is a need especially when the author disagrees with certain comments provided by reviewer(s).

5. The CEE sends the revised paper out for re-review. Typically, at least one of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the CEE in consultation with the EiC and EBMs examine their comments and decide whether the paper is ready to be published, needs another round of revisions, or should be rejected.

7. If the decision is to accept, an acceptance letter is sent to all the author(s), the paper is sent to the Press. The article should appear online in approximately three months.

   The Publisher ensures that the paper 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 Publisher. 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 article appears in the pages of the Journal and is posted online.

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# TROPICAL AGRICULTURAL SCIENCE

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# JTAS

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**Pertanika Journal of Tropical Agricultural Science**
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# Foreword

Welcome to the Second Issue of 2020 for the Journal of Tropical Agricultural Science (JTAS)!

JTAS 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 10 articles; 2 are review articles, 1 is short communication and the rest are regular articles. The authors of these articles come from different countries namely Malaysia, Mexico, Nigeria and Thailand.

Articles submitted in this issue cover various scopes of Tropical Agricultural Science including: animal production; aquaculture; crop and pasture production; fisheries sciences; genetics and molecular biology; plant physiology; soil and water sciences; veterinary sciences; and zoology.

An investigation to determine the antifungal potential of *Andrographis paniculata*, *Backhousia citriodora*, *Clinacanthus nutans*, *Ficus deltoidea*, *Phaleria macrocarpa*, and *Piper betle* against three economically-important fungal pathogens was conducted by Mui-Yun Wong and her colleagues from Universiti Putra Malaysia. The results showed that *P. macrocarpa* was most potential to be developed as a biofungicide. For the development of novel biofungicides, further experimental investigation into bioactive compounds of these herbal plants as well as trials in both glasshouse and field are strongly recommended. The detailed information of the article can be found on page 107.

Muslim Razani and his teammates from Universiti Kelantan Malaysia focused on somaclonal variation caused by long-term subculture. The morphology changes of the micropropagated *Musa acuminata* cv. Berangan plantlets from long-term subcultures ($15^{th}$ subculture) could be differentiated by RAPD pattern. They found out that the micropropagated banana cv. Berangan produced different plant heights which were not uniform. This phenomenon is related to the genetic changes which can be proven by using RAPD analysis. Further details of this study is presented on page 183.

A regular paper entitled "Antipyretic Effect of Mitragynine and Crude Methanolic Extract of *Mitragyna speciosa* Korth. in Mice" discussed on the antipyretic effect of mitragynine and methanolic extract of *Mitragyna speciosa* Korth. (MSM) using mice as an *in vivo* pyretic model. It concluded that both of them possessed a dose-dependent antipyretic effect, where intraperitoneally (i.p.) administration of MSM resulted in an opioid-like effect. Details of this study are available on page 207.

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 JTAS, who have made this issue possible.

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

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*Review article*

# Salt Tolerance Research in Sago Palm (*Metroxylon sagu* Rottb.): Past, Present and Future Perspectives

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## ABSTRACT

The sago palm (*Metroxylon sagu* Rottb.) is one of the underdogs in the food crop planting industries for its potential which is extremely vast but the community appreciating it is scarce. Its capabilities to thrive well in undesirable environmental conditions, salt tolerance and high starch yield are one of the many advantages it possesses over other food crops like wheat, corn and rice. One important factor to look into for crop plantation is none other than its salt tolerance. The salt tolerance researches on this unique palm have commenced since 1977 and the pace of research was unbelievably slow in progression. Nevertheless, it was not until recently that this palm was being placed in the limelight once more. In this review, we are focusing on salt tolerance research and further detailed on the past, present and future of this research line. It is anticipated that consolidation of talents and resources can come in time and in tandem for the utilization of this cash palm to end world hunger.

*Keywords*: Food crop, food security, sago palm, salt tolerance, starch yield

## INTRODUCTION

The sago palm (*Metroxylon sagu* Rottb.) is a true palm Calamideae subfamily member categorized under the order Arecales and family Arecaceae. This palm is native to Southeast Asia countries like Malaysia, Philippines, Papua New Guinea and Indonesia, thriving well in tropical rain forests as well as low-land freshwater swamps (Johnson, 1977). The sago palm is a hapaxanthic (only flowers once per stem) multiple-stemmed type of palm and its flowers emerge from the upright terminal of its 10 to 15 metres stem (Kiew, 1977; Kueh, 1977).

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The sago palm undergoes a period of stemless vegetative growth with 175 cm leaf blades extending from its base (Lim, 1991). Upon elongating its stem up to its maximum height, its once-per-lifetime inflorescence develops where spirally arranged flower pairs emerge from the third-order branches encompassing a male and hermaphrodite flower prior to fruiting (Kueh et al., 1987; Lim, 1991). The palm reaches the end of its lifecycle upon bearing fruit and usually the best time to harvest the heavily starch-packed trunk is before the flowering stage in which the starch content is maximal (Hamanishi et al., 1999; Lim, 1991; Pei-Lang et al., 2006). The trunk of the sago palm is not the only plant organ with high economic value (Ehara, 2009; Husaini et al., 2016; Karim et al., 2008), the list goes from the leaf petioles, fronds, pith, sap and down till the fibrous residue ("*hampas*"), all of them playing major roles in industries like renewable energy, textile, pharmaceutical, food as well as polymer (Ishiaku et al., 2002; Nuttanan et al., 1995; Purwani et al., 2006; Radley, 1976). In the field of research, the sago palm is one palm that is progressively moving into the limelight, tapping into fields like microbiology, plant biology, sago waste management, bioprocess technology, computational biology, genetics and phenotypic studies (as reviewed by Lim et al., 2019c).

One of the most famous investigations in the fields of plant biology, physiology and genetics is no other than the salt tolerance research. Salt tolerance research, or widely known as salinity test or salt stress investigations, is one important field of research to determine the survivability of plants (especially essential food crops) against salt-inducing environment. Under salt stress, plants generally tend to cease in terms of water and essential elements absorption as well as photosynthesis due to the sudden plummet in water potential in soil and turgor pressure in cells (Kaku, 1996). Almost all crops suffer from mortality at a threshold NaCl level of 100 mM and beyond, however halophytic plants like the sago palm is vastly known to have the capability to withstand this salt stress using its exclusively equipped mechanisms (Matoh, 1999). Some halophytic plants like the sago palm can uptake salts from the root system to prevent metabolic interruptions via the cancelling of osmotic pressure gradient between cytoplasm and vacuoles as a result of high salt level (Matoh, 1999). Osmoregulating compounds, at this time, play their roles in restoring the osmotic pressure gradient and those who do not disrupt the metabolic activities when present in high concentrations are known as compatible solutes and they are usually low in molecular weight (Nakamura & Takabe, 1999). Some of the well-known compatible solutes are proline, sugar alcohols, glycine betaine and inorganic ions (Wada, 1999). Besides, $K^+$ absorption mechanism is also found in the sago palm, although it is rarely found in other crops, facilitating it in terms of selective $K^+$ absorption (Tadano, 1986; Yoneta et al., 2006). These mechanisms involve various metabolic pathways and genes in the sago palm, in which our

knowledge on these are very much limited and still in its infancy. In this review, we place-focus on salt tolerance research and further detailed on the past, present and future of this research line in hope to consolidate talents and resources in the utilization of this cash palm to end world hunger.

**Impact of Soil Salinity on Sago Palm**

The sago palm naturally inhabits along the banks of rivers, swamps and lakes, is equipped with strong adaptability towards land from sea level up to 1.2 km elevation (Schuiling, 2009). The optimal growth habitat for this palm is below 0.4 km elevation where its growth performance (speed and yield) starts to decline at elevations exceeding the abovementioned altitude (Djoefrie, 1999). A much more miniature trunk diameter and diminished height (6 metres difference) was observed on sago palm trees planted at above 0.6 km elevation (Schuiling & Flach, 1985).

A vast variety of soil can support the growth of the sago palms and they are divided into developed and undeveloped soils (Notohadiprawiro & Louhenapessy, 1992). Developed soils like tropohemists and sulfihemists, tropaquepts, thaptohistic fluvaquents as well as troposaprists of peatlands are proven conducive for sago palm growth (Notohadiprawiro & Louhenapessy, 1992). The psammaquents, hidraquents, fluvaquents, sulfaquents and tropaquents are undeveloped soils that can sustain optimal sago palm growth (Notohadiprawiro & Louhenapessy, 1992). Under swampy conditions, sago palms can thrive well in concentrated levels of organic matter and mineral nutrition, without pneumatophores being submerged, as well as marginally acidic brownish stagnant water, all in which favours the propagation of microbiota benefiting sago palm development (Bintoro et al., 2010). In swampy areas situated in close proximity to the ocean, these palms are hardy towards salinity, especially sodium ions where excess sodium ions are stored in the root organ. The photosynthetic activities of the sago palms are not affected up to the salt concentration of 0.2 M (Yoneta et al., 2006).

Moreover, soils with podzolic, alluvial, volcanic, hydromorphic and grumusol properties are all suited to the growth betterment of the sago palms (Djoefrie, 1999); provided that the optimal amount of microbiota and nutrients (potassium, phosphorus and magnesium) are abundantly available (Haryanto & Pangloli, 1992). On the side note, starch synthesis can be easily halted under over-waterlogged environmental conditions (Haryanto & Pangloli, 1992). The peatlands, consisting of great level of organic matter (C-organic > 18%) and more than half a metre organic matter depth, are also supportive of the sago palm optimal growth (Agus & Subiksa, 2008). Majority of sago palms in Malaysia are planted on peatlands but their productivities are very much affected by the lack of minerals in soil. For instance, the time of harvest can be shortened by 2.9 years if the palm was planted in mineral soil as compared to peat soil (Kueh et al., 1991).

Besides, the leaf amount of sago palm is 4% to 35% fewer when it is planted in peat soil than those planted in mineral soil (Bintoro, 2008; Flach & Schuling, 1991). Indirectly, the dry starch content of sago palm is found to have diminished by 29.6% to 82.1% when planted in peat soil in comparison to mineral soil (Sim & Ahmad, 1991).

The period for the initiation of maturity phase (rosette phase) in sago palm tends to be lengthy than usual when it is being subjected to suboptimal growth conditions, delaying the emergence of new leaves and starch synthesis (Flach et al., 1986). According to Bintoro et al. (2018), the high salt and hydrological tolerance of the sago palms have granted them great economic advantage over other food crops beside the high yield as they can literally thrive in almost all harsh environments where other plants can hardly survive.

**Salt-adaptation Capacity of Sago Palm**

The sago palms, like other halophytic plants, are naturally equipped with specific mechanisms to orchestrate salt uptake and strengthen their immunity against adverse salt effects, enabling them to thrive with brackish waters (Okazaki & Sasaki, 2018). Back in the year 1977, Flach et al. (1977) was one of the first to initiate investigations on the salinity of sago palms and it was found that this palm can survive in solutions with up to 12.5% to 14.3% of electrical conductivity (which corresponds to salinity) to that of seawater where any concentrations beyond had declined in the growth of sago palms.

Generally, terrestrial plants experience salt stress when exposed to a salt level of 0.1% (around 20 mmol $L^{-1}$) and growth ceases significantly when the level is beyond 0.3% (Matoh, 2002). However, it was discovered that sago palm seedlings depicted the best growth pattern at salt concentration of 0.05% (around 10 mmol $L^{-1}$) NaCl solution, especially when compared to 0% NaCl solution (Yoneta et al., 2004, 2006). It was not until the salt concentration of around 50 to 200 mmol $L^{-1}$ (0.25% to 1.0 %) that the growth performance started to deteriorate (Yoneta et al., 2004, 2006). There are two types of salt stress, namely ionic stress and osmotic stress (Okazaki & Sasaki, 2018). The ionic stress is the primary factor of salinity stress where it involves the excess mutilation induced by salt-composing ions that pose specific physiological adverse effects (Okazaki & Sasaki, 2018). On the other hand, osmotic stress refers to the hindrance of water uptake induced by the osmotic pressure due to the presence of high salt level from the environment (Okazaki & Sasaki, 2018).

The sago palm is a special food crop with incredibly high salt tolerance and the following are some of the known mechanisms against salt stress. Sago palm tends to transport excess $Na^+$ ions from the roots to the leaflets and petioles when being subjected to 0% to 0.2% NaCl solution but it is not the case for sago palm seedlings as seedlings prefer to stockpile $Na^+$ ions within the roots and progressively transporting these ions to the lower leaves (Ehara et al., 2006). The transpiration rate of the sago

palm was also found to decrease with the elevating levels of NaCl in the treatment solution and at the same time the level of potassium ions in the petiole rises (Ehara et al. 2008). In order to sustain the high osmotic pressure of the cytoplasm, sago palm employs a brilliant strategy to surge the uptake of compatible solutes like glycine betaine, potassium ion and proline to function as orchestrator of osmotic pressure (Yoneta et al., 2006). Furthermore, the sago palm was also discovered to have a unique way to halt the absorption of excess $Na^+$ ions via its special barriers (Ehara et al., 2003, 2006).

**The Past**

The past researches involved the investigations on salt tolerance capability of the sago palm in terms of plant biology and physiology. In other words, the sago palm is tested on their response to NaCl physically and chemically. One of the first salinity investigations on sago palm was that by Flach et al. (1977) where they discovered that the growth of the sago palm was not halted in a Hoagland solution with six to seven millisiemens (mS) per cm electrical conductivity. This electrical conductivity unit is equivalent to around one eighth to one seventh to that of seawater (Flach et al., 1977). A study by Hirotsu et al. (2002) unravelled that proline and glycine betaine were produced in both *Metroxylon* genus counterparts, namely *M. sagu* and *M. warburgii* when they were subjected to salt stress. Similarly, it was discovered that *M. sagu* tended to synthesise 0.16 to 3.0 mg $kg^{-1}$ dry weight of glycine betaine when induced by salt concentration ranging between 0 to 200 mM (Yoneta et al., 2003).

In the year 2006, Yoneta et al. (2006) performed a NaCl stress investigation by immersion of sago palm seedlings grown in 5 L plastic pots into 6 L plastic pots at various salt concentrations, namely 0, 10, 50, 100, 200 and 400 mM. The greatest growth of sago palm was in the ones being subjected to 10 mM NaCl and growth was stunted at concentrations beyond 50 mM (Yoneta et al., 2006). In this study, the highest glycine betaine was found in the 200 mM NaCl treatment set, and overall there was no correlation between glycine betaine and NaCl level (Yoneta et al., 2006). Ehara et al. (2006) compared the salt accumulation in different organs of the sago palm seedlings, namely root, petiole and leaflet, between treated (86 mM or 0.5% NaCl) and non-treated samples. The $Na^+$ accumulation in the roots was found to be 5.3 to 6.6 times that of the negative control whereas the petioles were discovered to store 1.7 to 8 times more $Na^+$ than the negative control (Ehara et al., 2006). Generally, the lower positioned petioles stored more $Na^+$ than the upper positioned petioles in both negative control and treated samples (Ehara et al., 2006). The leaflets $Na^+$ accumulation was found to be 4 times higher in treated samples as compared to negative controls and they were all had lower levels of $Na^+$ in comparison with petioles in all positions (Ehara et al., 2006). However, the $K^+$ level tended to be higher in upper positioned petioles and leaflets than the lower ones.

Despite the differences, the correlation between $K^+$ and $Na^+$ concentrations was not significant (Ehara et al., 2006). In the same study, Ehara et al. (2006) tested the sago palm seedlings with different NaCl concentrations (0.5%, 1.0% and 2.0% NaCl) and yet the $K^+$ concentration change was negligible. The transpiration rate of samples treated in 2.0% NaCl was also found to be 65% that of the negative control set (Ehara et al., 2006). They concluded that the sago palm exhibited avoidance salt tolerance mechanism in which it maintained low $Na^+$ in the leaflets by stocking $Na^+$ in the roots and petioles instead (Ehara et al., 2006).

Ehara et al. (2008) utilized sago palm seedlings at $8^{th}$ or $9^{th}$ leaf stage ($9^{th}$ or $10^{th}$ leaf emerging) and subjected them to treatment of 342 mM (2.0%) NaCl for 31 days. Similar to previous investigation (Ehara et al., 2006), a low $Na^+$ level was regulated in the sago palm leaflets and higher $Na^+$ accumulations were uncovered in the roots and petioles (Ehara et al., 2008). The $Na^+$ was found to be higher in the cortex than in the stele, in the large roots following NaCl treatment, and Ehara et al. (2008) suspected the role of endodermis in orchestrating the $Na^+$ influx between the stele and cortex. They further examined the endodermis via X-ray micro-analysis and unveiled the fact that there was dense distribution of Na in close proximity to the large root endodermis (Ehara et al., 2008). It was also been revealed in the same study that the $Ca^{2+}$ and $Mg^{2+}$ changed in relation to that of $Na^+$ were miniature (Ehara et al., 2008). Despite the slight postponement in new leaves emergence and great decline in the rate of transpiration, the dry matter weight of all petioles and leaflets (regardless of position) had depicted no significant differences (Ehara et al., 2008). They further concluded that, apart from the avoidance mechanism previously proven in Ehara et al. (2006), the sago pam tended to impose constraint on transpiration by locking the water in the leaves (Ehara et al., 2008, 2011).

In the year 2011, Prathumyot et al. (2011) extended the salinity investigation further by conducting two cycles of diurnal NaCl concentration alterations (in the order of 224 mM, 0 mM, 224 mM and 0mM NaCl) for four months in a hydroponic system using four spiny sago palm seedlings (two treated samples and two negative controls). Interestingly, the amount of dead leaves in treated palms mirrored that of the control palms despite that the emergence of new leaves was much slower in treated samples (Prathumyot et al., 2011). They further tested on the phosphorus and nitrogen levels in the petioles and leaflets at all positions and unravelled that both phosphorus and nitrogen levels did not vary with NaCl treatments (Prathumyot et al., 2011). Moreover, the increment rate of chlorophyll level was found to be slower in the treated palms (measured in SPAD value) as compared to their control counterparts, together with the rate of photosynthesis, transpiration as well as stomatal conductance which declined by around 40% (Prathumyot et al., 2011). The $Mg^{2+}$ levels were also tested, and a huge diminished level was observed in the

cortex, but the levels were not significant in leaflets, adventitious roots as well as petioles (Prathumyot et al., 2011). They concluded that the translocation of macronutrients like potassium, nitrogen and phosphorus was not affected by the induced NaCl stress in sago palm, and that chlorophyll synthesis was slowed down but not stunted, displaying the ability of sago palm to resist salt stress albeit the slower growth rate, without signs of mortality (Prathumyot et al., 2011).

**The Present**

The present researches on salt tolerance of sago palm are more focused on the genetic and genomic field due to the booming of next-generation sequencing technologies. Roslan et al. (2017) had isolated and fully characterized the fructose-1,6-bisphosphate aldolase gene from *M. sagu* at the level of DNA, RNA and protein, which the protein of this gene was found to increase the salinity tolerance of *Escherichia coli*. On the other hand, Lim et al. (2020) had successfully sequenced the entire chloroplast genome of *M. sagu*, which provided huge insights on all salinity response genes from the chloroplast of the sago palm and opened new windows for their comparisons across other palms and food crops.

In the year 2017, Roslan et al. (2017) had successfully isolated full-length sequences of the fructose-1,6-bisphosphate aldolase gene via polymerase chain reaction (PCR). Furthermore, they also isolated the full-length cDNA of this gene via reverse transcriptase PCR (RT-PCR) and rapid amplification of cDNA ends PCR (RACE-PCR) subsequently. In order to synthesise the protein of this gene, a pET41a(+) expression vector was chosen to be transformed into *E. coli* BL21 (DE3) strain (Roslan et al., 2017). The recombinant protein was then isolated via the nickel column which bound to the histidine affinity tag present at the fused target protein. Upon analysis, the gene was revealed to have 2322 bp in nucleotide length with five exons discovered (Roslan et al., 2017). The predicted protein of this gene has a molecular mass of 39.14 kDa and an isoelectric point of 6.49 (Roslan et al., 2017). They further characterized the fructose-1,6-bisphosphate aldolase protein via functional assay on *E. coli* (containing the recombinant pET41a(+) expression plasmid) treated with various NaCl concentrations, namely 0, 0.2, 0.4, 0.6, 0.8 and 1.0 M (Roslan et al., 2017). As a result, the survivability (in terms of colony count) of *E. coli* at 1.0 M NaCl as compared to 0 M NaCl in treated samples reduced by only 35% but the drastic reduction (85%) was observed in the negative controls (containing empty pET41a(+) plasmid) (Roslan et al., 2017). They concluded that this recombinant protein of fructose-1,6-bisphosphate aldolase gene had provided salinity immunity towards the tested *E. coli* cells (Roslan et al., 2017).

Recently, Lim et al. (2020) had sequenced and characterized the entire chloroplast genome of *M. sagu* (GenBank accession number: MN309778). Various genomic analyses such as codon usage, microsatellite, long repeats, inverted repeat structure (expansion and contraction), RNA

editing and phylogenetic analysis were conducted and reported in detail (Lim et al., 2020). The chloroplast genome of *M. sagu* was revealed to have high similarities to chloroplast genomes of other palms from the same family (Arecaceae) (Lim et al., 2020). The *M. sagu* closely resembles that of its genus counterpart, *M. warburgii* with high similarity over 98% (Barrett et al., 2016; Lim et al., 2020). To name a few, other palms with relatively high similarity to that of the *M. sagu* chloroplast genome includes *Phoenix dactylifera*, *Pigafetta elata*, *Mauritia flexuosa*, *Elaeis guineensis* and *Eugeissona tristis*.

## The Future

The future researches on salt tolerance of sago palm will surely be the upgrade of both past and present researches, which include the identification of new compounds responsible for salinity as well as characterization of more salinity response genes from the *M. sagu* genome, transcriptome and proteome.

**Genetic and Epigenetic Studies.** One of the most studied palm counterparts of *M. sagu* in terms of salt tolerance is the none other than *P. dactylifera* or widely known as the date palm. There are several aspects of salt tolerance researches that the *M. sagu* are lacking behind *P. dactylifera*, namely genome-wide miRNAome related to salinity, genome-wide salinity response expression profiling, growth improvement, antioxidant response to salinity, differential DNA methylation profile related to salinity, identification of set of salinity response genes as well as salinity related proteome analysis (Al-Harrasi et al., 2018; Al Kharusi et al., 2019; Darwesh, 2013; El Rabey et al., 2016; Patankar et al., 2018; Yaish et al., 2017). Undoubtedly, with these efforts being implemented onto sago palm trees, it is not daunting to reveal the contributing factors towards the salinity tolerance of sago palm, be it in terms of novel salt-induced motifs, overexpression or downregulation of several salt stress genes as well as novel salt-deterring protein products.

**Morphological and Variation Studies.** Most importantly, the core of these researches are to enable the deciphering of salinity adaptation mechanism of the sago palm. A good starting point and foundation would be the characterization of physical and morphological properties of the palm, encompassing histology, ion transport, growth rate, tissue content and many more (Faiyue et al., 2012; Gong et al., 2006; Yaish & Kumar, 2015; Yeo et al., 1999). This would help to first establish and identify salt-tolerant varieties to start with. For instance, there was a study conducted by Al Kharusi et al. (2019) which had tested on the antioxidant response of two varieties of date palms ("Umsila", the salt tolerant variety and "Zabad", the salt susceptible variety) towards salinity and as a result, the salt tolerant date palm variety was found to have higher reactive oxygen species (ROS)-scavenging metabolites and a balanced $Na^+$ and $K^+$ uptake. This type of experimentation can be easily inculcated on

the sago palm to further elucidate on this aspect of study for comparison purposes and knowledge enrichment. Furthermore, these characterization efforts of the sago palm variants would definitely provide a strong foundation and reservoir for further genetics, phylogenetics, taxonomical and genomics analysis.

**Growth Improvement Studies.** Next, several growth improvement research can be conducted onto the sago palm to achieve the maximum growth and yield, like the one published by Darwesh (2013) on date palm where the author reported on the combinations of yeast and amino acids (40 and 50 mL/L yeast as well as 3.0 and 6.0 mL/L amino acids) that worked best in alleviating the adverse salt stress effects in date palm. With these growth improvement studies being initiated on sago palm in the future, it would greatly help sago palm cultivators to have confidence and grasp on the knowledge to yield maximum profit from the palm plantation as well as ways to curb salt invasion issues in sago palm trees.

**Genomics Studies.** With the advancement of the sequencing technology throughout decades, the genomics of higher plant species can be easily unravelled with much lower costs and processing time with higher throughput and quality. The chloroplast genome of the sago palm has been recently sequenced and characterized (Lim et al., 2020) but the sequences of the nuclear and mitochondrial genome remained unknown to date. As nuclear and mitogenomes are crucial in terms of the comprehension of the salt stress mechanisms, these missing puzzle pieces are no qualms beneficial in explaining the superior salinity tolerance of this unique sago palm. With the booming of mitogenomes and nuclear genomes being published at an exponential rate to date, the focus of research has now moved towards the various non-model plant organisms such as the early flowering plant *Nymphaea colorata* (Dong et al., 2018), Norway spruce (Sullivan et al., 2020), *Chrysanthemum boreale* (Won et al., 2018), legume *Vicia faba* (Negruk, 2013), sugarcane (Evans et al., 2019) and many more. The information obtained from the complete sequences of nuclear and mitogenomes of sago palm will in turn aid in selective breeding, variation studies as well as salt stress mechanism pathways (Lim et al., 2019d).

**Transcriptomic and Proteomic Studies.** With the aid of the next generation sequencing technologies, the OMICS of these sago palm varieties will aid in yielding information on proteins or other compounds that orchestrate salt transport like the results achieved by El Rabey et al. (2016) on date palm where various proteins related to drought and salt resistance were discovered. The genome-wide expression profiling in leaves and roots of date palm exposed to salinity as reported by Yaish et al. (2017) was another interesting aspect of study to be implemented on the sago palm because a sum of 4687 and 2630 date palm genes were identified to be differently expressed under salt stress and the potential of sago

palm to be included into similar study like this is limitless. Recently, the differential methylome and transcriptome of date palm in response to salinity had been drafted by Al-Harrasi et al. (2018) and this will be highly reproducible in sago palm. Emulating the success of Patankar et al. (2018) in which a total of 24 salinity response genes were identified and functionally characterized in date palm, it is not impossible to produce a comparable gene list from that of sago palm. These would surely contribute to the salt related gene landscape formation of sago palm which will in turn help in the field of genetic engineering and selective breeding. The genome-wide identification of enhancers involved in salinity response mechanism in sago palm is also possible with the utilization of computational tools as reviewed by Lim et al. (2018a).

## CONCLUSION

The sago palm is one of the trees of life for its many uses ranging from food, textile, polymer, pharmaceutical, renewable energy and its role in the environment (carbon dioxide absorption and water conservation). Adding to its many advantages is that it is one of the most important food crops in future (yielding at least three-fold to that of our current established food crop), and it can be cultivated in order to achieve the ultimate goal of combating global food scarcity problems. The salt tolerance research on this palm is essential to improve our comprehension on what distinguishes it from other food crop in terms of genetic aspect and physiology, besides providing us with the idea to incorporate these novel sago palm salinity resistant specific motifs in the field of genetic engineering to further improve the feasibility of other food crop to curb salt stress in future. These researches will also provide us with the knowledge and resources in time to establish its status and expand its cultivations worldwide, besides enriching the research database for endemic fauna and flora in Sarawak, Malaysia (Kadir et al., 2013; Lim et al., 2018b, 2019a, 2019b; Ministry of Natural Resources and Environment [MNRE], 2016; Phillips, 2016; Soepadmo & Wong, 1995), as well as in enabling the study of ecosystem wholly for aiding future conservation efforts. In short, as this cash crop is one expensive thing to be wasted and remain undiscovered, consolidated research efforts should start right now.

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## CONFLICT OF INTEREST

The authors declared no conflict of interest.

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# Antifungal Potential of Six Herbal Plants against Selected Plant Fungal Pathogens

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## ABSTRACT

An investigation was conducted to determine the antifungal potential of *Andrographis paniculata*, *Backhousia citriodora*, *Clinacanthus nutans*, *Ficus deltoidea*, *Phaleria macrocarpa*, and *Piper betle* against selected plant fungal pathogens. Dilutions of crude leaf extracts (5, 10, 15, and 20%) of these plants were screened *in vitro* against *Ganoderma boninense*, *Fusarium oxysporum* f. sp. *cubense* R4 (*Foc*R4), and *Rhizoctonia solani*. The percentage inhibition of diameter growth (PIDG) of test pathogens was measured using poisoned agar method. Aqueous extract of *A. paniculata* was ineffective in inhibiting the mycelial growth of test pathogens at all test concentrations while that of *B. citriodora* markedly inhibited *Foc*R4 growth at 15% (PIDG 70%) and 20% (PIDG 72.9%) concentrations. Methanol extract of *C. nutans* at 20% concentration significantly inhibited *R. solani* growth (PIDG 64.4%) meanwhile that of *P. betle* at 20% considerably inhibited the growth of *Foc*R4 (PIDG 94%), *G. boninense* (PIDG 89.4%), and *R. solani* (PIDG 82.8%). Complete inhibition (PIDG 100%) of *G. boninense* and *R. solani* was obtained at 10% concentration of *F. deltoidea* and *P. macrocarpa* methanol extracts. Leaf extracts of five herbal plants have the potential to be used as bio-fungicides as a safe alternative to synthetic fungicides.

*Keywords*: Biopesticide, botanical extract, green technology, poisoned agar method

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## INTRODUCTION

Immoderate and improper use of synthetic fungicides, which are broadly used in the management of fungal diseases in plants, adversely affects the human health and the environment. Use of plant extracts with antimicrobial properties is therefore regarded as a safer approach in controlling plant fungal diseases. Plants synthesize an assortment of antimicrobial compounds including alkaloids, flavonoids, terpenes and phenolic compounds (Compean & Ynalvez, 2014). Recently, numerous studies have assessed the efficacy of plant extracts in inhibiting the growth of some economically-important plant pathogens. For instance, Satish et al. (2009) had reported the substantial antifungal activities of 12 plants against 8 *Fusarium* species. Besides, the leaf extract of *Artemisia absinthium* L. has been reported to possess antifungal activities against rot-causing pathogens namely, *Alternaria alternata*, *Mucor piriformis*, and *Penicillium expansum* (Parveen et al., 2014). On the other hand, 3 out of 39 plant extracts had been proven effective against *Alternaria solani* (Ravikumar & Garampalli, 2013). Up to now, little attention has been paid to the antifungal activity of plants from Acanthaceae, Myrtaceae, Moraceae, Piperaceae, and Thymelaeaceae. The antifungal potential of plant extracts from these families against a broad spectrum of plant fungal pathogens including *G. boninense*, *F. oxysporum* f. sp. *cubense* R4 (*Foc*R4), and *R. solani* is lacking. The negative impacts of these soilborne fungal pathogens on production of some economically-important crops such as oil palm, banana and rice have been vastly reported. For instance, *G. boninense* is the major pathogen causing the deadly basal stem rot in oil palm where the annual loss caused by this pathogen has been estimated as USD500 million (Arif et al., 2011). Meanwhile, *Foc*R4 is the causal agent of Fusarium wilt in an assortment of banana cultivars including Cavendish with the estimated annual losses ranging from USD14.1 to 253.3 million (Malik et al., 2013; Peng et al., 2013). On the other hand, rice sheath blight caused by *R. solani* is one of the major threats to global rice production where the estimated annual losses of 20% and 10% have been reported in Thailand and India, respectively (Boukaew & Prasertsan, 2014). Therefore, urgent consideration is needed to develop effective and sustainable approaches to control these diseases. Identification of plant extracts with antimicrobial activity could be useful for development of novel biopesticides against these pathogens. The present study explores, for the first time, the effects of leaf extracts of *A. paniculata*, *B. citriodora*, *C. nutans*, *F. deltoidea*, *P. macrocarpa*, and *P. betle* against *G. boninense*, *Foc*R4, and *R. solani*.

## MATERIALS AND METHODS

### Collection of Plant Leaves

The leaves of *A. paniculata*, *C. nutans*, *F. deltoidea*, and *P. betle* were collected from Herb Garden, University Agriculture Park, Universiti Putra Malaysia (UPM), Serdang, Selangor. Meanwhile, the leaves of *B. citriodora* and *P. macrocarpa* were collected

from Department of Agriculture, Serdang, Selangor. The experiments were carried out in Mycology Laboratory, Department of Plant Protection, Faculty of Agriculture, UPM, Serdang, Selangor. The leaves of *C. nutans*, *F. deltoidea*, *P. macrocarpa*, and *P. betle* were thoroughly washed and air-dried at room temperature (26±2°C). The leaves of *A. paniculata* and *B. citriodora* were treated in the same manner but drying was done at 60°C. All the dried leaf samples were ground separately to fine uniform texture using grinder (Retsch Model SK 100) and stored at room temperature (26±2°C) until use.

**Fungal Cultures**

The test fungal cultures namely, *G. boninense* (UPMGB002), *F. oxysporum* f. sp. *cubense* R4 (*Foc*R4) (UPMFTR4-01), and *R. solani* (UPMRS01) were obtained from the Department of Plant Protection, Faculty of Agriculture, UPM, Serdang, Selangor. *Foc*R4 and *R. solani* were sub-cultured and maintained on potato dextrose agar (PDA). Meanwhile, *G. boninense* was cultured on malt extract agar (MEA). These cultures were maintained in the culture chamber under laboratory condition.

**Preparation of Leaf Crude Extracts**

Fifty grams of ground *A. paniculata* and *B. citriodora* leaves were separately soaked in 300 ml distilled water and stirred at 120 rpm for 24 h using an orbital shaker. The leaf crude extract of each plant was collected through filtration using Whatman No-1 filter paper. Then, the solvent was evaporated using Buchi Rotavapor Model R215 (BÜCHI Labortechnik AG, Flawil, Switzerland). The dried extract was collected in an air-tight container and stored at 4°C. The same method was used in preparation of leaf crude extracts of *C. nutans*, *F. deltoidea*, *P. betle*, and *P. macrocarpa*, however 300 ml methanol was used as described by Venkateswarlu et al. (2013).

**Screening of Antifungal Activity**

The concentrations of leaf crude extract tested in the antifungal test were 5, 10, 15, and 20% (v/v). The stock solutions of the leaf crude extract of *A. paniculata* and *B. citriodora* were prepared separately by diluting the crude extract of each plant with distilled water at the ratio of 1:1 as described by Dadang and Ohsawa (2001). The stock solution of *P. macrocarpa* crude extract was prepared by diluting the leaf crude extract with acetone at the ratio of 1:10 as described by Dadang and Ohsawa (2001). On the other hand, the stock solutions of *C. nutans*, *F. deltoidea*, and *P. betle* extracts were prepared separately by diluting the leaf crude extract of each plant with methanol at the ratio of 1:10. Further serial dilution was done to achieve test concentration. Petri dishes containing 15 ml of poisoned medium were used. Then, a respective fungal plug (0.4 cm diameter) was placed at the centre of a PDA plate containing leaf extract of each plant at the defined concentrations except for *G. boninense* where MEA plates containing leaf extract of each plant at the defined concentrations were used. The antifungal activity of *A. paniculata*, *B. citriodora*, *C.*

*nutans*, *F. deltoidea*, *P. macrocarpa*, and *P. betle* extracts were separately tested against *G. boninense*, *Foc*R4, and *R. solani*. The plates were incubated at room temperature (26±2°C) until the mycelial growth in control plates for certain fungal species had reached the edge of the plates. The colonial diameter was measured daily and percentage inhibition of diameter growth (PIDG) values was calculated using Equation [1] as described by Lee et al. (2018).

$$PIDG = \frac{D1 - D2}{D1} \times 100\% \qquad [1]$$

where;
D1 = Diameter growth of mycelia in control plates
D2 = Diameter growth of mycelia in treatment plates

**Experimental Design and Statistical Analysis**

The *in vitro* screenings of antifungal potential of all test plants were conducted in complete randomized design (CRD) with 5 treatments (0, 5, 10, 15, and 20%). There were at least 3 replicates for each treatment. Statistical analysis was conducted using SAS® Software (SAS Institute, North Carolina, USA, Version 9.4, 2012) and comparison of means using Least Significant Difference (LSD) at 5% probability level.

## RESULTS AND DISCUSSION

The antifungal activity of *A. paniculata*, *B. citriodora*, *C. nutans*, *F. deltoidea*, *P. macrocarpa*, and *P. betle* extracts differed when tested against *Foc*R4, *G. boninense*, and *R. solani*. According to Kurucheve et al. (1997), the variation in the inhibitory effect of plant extracts is the results of the qualitative and quantitative differences in antifungal properties of the extracts.

As presented in Figure 1, the aqueous extract of *A. paniculata* was ineffective in inhibiting the mycelial growth of *G. boninense*, *Foc*R4, and *R. solani* at any test concentrations. The leaf extract of *A. paniculata* was previously reported to inhibit the radial mycelial growth of *F. oxysporum* (Neela et al., 2014). Furthermore, the antifungal potential of *A. paniculata* leaf extract also has been reported against *Fusarium verticillioides* (Yasmin et al., 2008) and *A. solani* (Nidiry et al., 2015). However, it was not the case in our study. This discrepancy could be attributed to the variations in the components of the *A. paniculata* extracts resulting from the different location and sample collection timing, storage and extraction conditions as suggested by Akbar (2011). For instance, Adegboyega and Oyewole (2013) had reported that the ethanol and methanol extracts of *A. paniculata* contained more phytochemicals as compared to the aqueous extract. Thus, a comparative study on the antifungal activities of *A. paniculata* leaf extracts obtained using different solvents against *G. boninense*, *Foc*R4, and *R. solani* is necessary to elucidate the *in vitro* antimicrobial effect of this plant against the test pathogens.

The aqueous extract of *B. citriodora* was significantly effective against *Foc*R4 at the concentration of 15% and above as

compared to other test pathogens (Figure 2). The concentration of 10% (PIDG 57%) was sufficient to inhibit the mycelial growth of this fungus. Hayes and Markovic (2002) had reported the notable antimicrobial activity of the essential oil extracted from *B. citriodora* and its key constituent namely citral, against an assortment of bacteria, yeast and fungi. In addition, the antimicrobial activity of 4 essential oils with varying citral content of this plant against 8 fungi and 13 bacteria have also been reported (Wilkinson et al.,

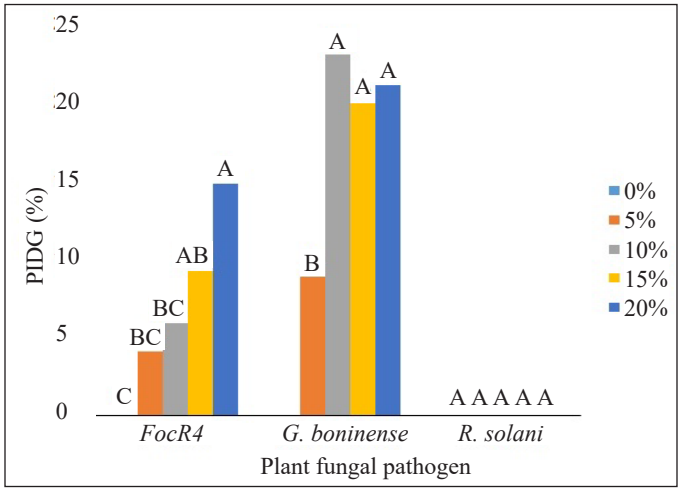


*Figure 1*. Percentage of inhibition of diameter growth of *Andrographis paniculata* against selected plant fungal pathogens. Measurement made at 11 days after inoculation (DAI) for *Fusarium oxysporum* f. sp. *cubense* R4 (*Foc*R4), 11 DAI for *Ganoderma boninense* and 5 DAI for *Rhizoctonia solani*. Values are the means of 6 replicates. Means with the same letter are not significantly different at P = 0.05

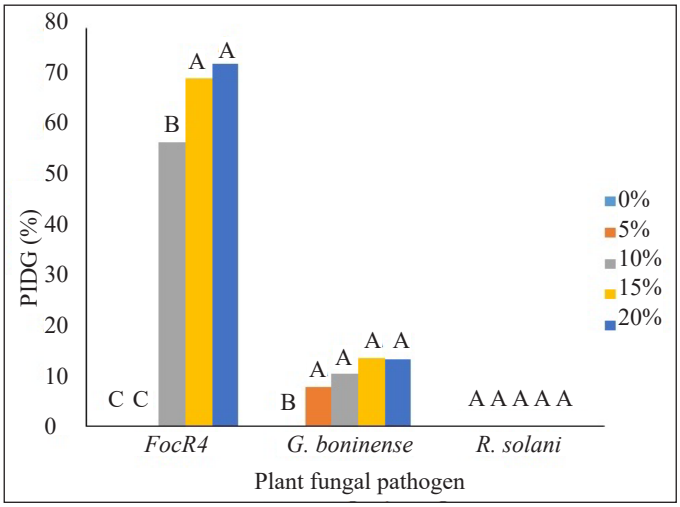


*Figure 2*. Percentage of inhibition of diameter growth of *Backhousia citriodora* against selected plant fungal pathogens. Measurement made at 11 days after inoculation (DAI) for *Fusarium oxysporum* f. sp. *cubense* R4 (*Foc*R4), 11 DAI for *Ganoderma boninense* and 5 DAI for *Rhizoctonia solani*. Values are the means of 6 replicates. Means with the same letter are not significantly different at P = 0.05

2003). However, the bioactive compounds accountable for antifungal effect of this plant extract against *Foc*R4 were not studied in the present study. To date, no reports of antifungal activities of *B. citriodora* against plant pathogens particularly *G. boninense*, *Foc*R4, and *R. solani* has been found in the literature. This study suggests that *B. citriodora* may serve as a good natural resource of new bioactive compounds for controlling *Foc*R4.

The methanol extract of *C. nutans* at 20% concentration significantly inhibited the mycelial growth of *R. solani* (PIDG 64.4%) as compared to *G. boninense* (PIDG 27.5%) and *Foc*R4 (PIDG 10.4%). Nevertheless, the concentration of 15% (PIDG 56.7%) was sufficient to inhibit the mycelial growth of *R. solani* (Figure 3). The presence of a broad range of bioactive compounds in this plant has been reviewed (Alam et al., 2016). So far, study of *C. nutans* has been only done to determine its antibacterial effect in aquaculture sector, pharmaceutical and medical sector (Arullappan et al., 2014). Use of *C. nutans* as a new source to control *R. solani* is highly recommended since *C. nutans* is widely available in Malaysia.

Strikingly, the methanol extracts of *P. betle* at 20% concentration significantly inhibited the mycelial growth of *Foc*R4 (PIDG 94%), *G. boninense* (PIDG 89.4%), and *R. solani* (PIDG 82.8%) as compared to all test concentrations (Figure 4). Nonetheless, the concentration of 15% was sufficient to inhibit the mycelial growth of *G. boninense* (54.1%) and *R. solani* (PIDG 71.9%). Likewise, effective antifungal activities of *P. betle* leaf extracts against *Aspergillus niger*, wild *Aspergillus* sp., and *Rhizopus* sp. (Pawar et al., 2017) as well as against *F. oxysporum* (Neela et al., 2014) have been reported. Previous studies had suggested that presence of the essential

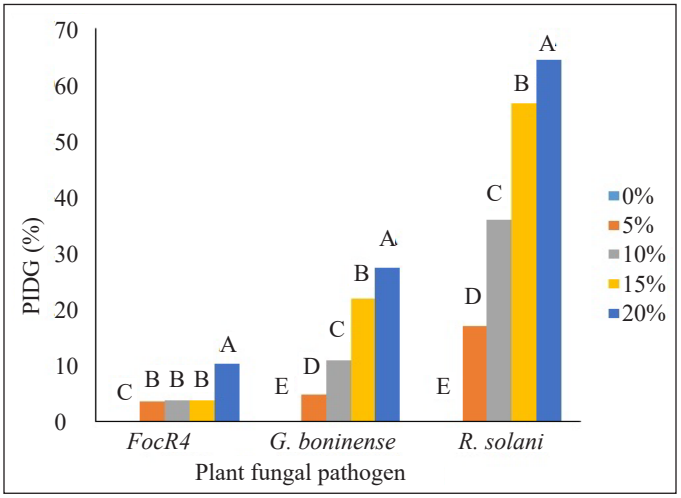


*Figure 3*. Percentage of inhibition of diameter growth of *Clinacanthus nutans* against selected plant fungal pathogens. Measurement made at 12 days after inoculation (DAI) for *Fusarium oxysporum* f. sp. *cubense* R4 (*Foc*R4), 12 DAI for *Ganoderma boninense* and 4 DAI for *Rhizoctonia solani*. Values are the means of 5 replicates. Means with the same letter are not significantly different at P = 0.05

oils which contained phenolic compounds in *P. betle* might contribute to inhibition of several phytopathogenic fungi (Ali et al., 2010; Begum et al., 2007). The results obtained in this experiment shed light on the potential use of *P. betle* in the management of several economically important plant diseases. Nevertheless, further analysis of the active compounds of this plant is highly recommended.

As shown in Figure 5, the methanol extracts of *F. deltoidea* significantly inhibited the mycelial growth of *G. boninense* and *R. solani* at all concentrations above 10%. However, the concentration of 5% was sufficient to inhibit the mycelial growth of *G. boninense* (PIDG 56%) and *R. solani* (PIDG 53.7%). To date, the antimicrobial activity of *F. deltoidea* leaf extract has been studied only on clinical pathogens (Abdsamah et al., 2012) and fish pathogen (Tkachenko et al., 2016). To our best knowledge, this experiment is the first study done on the plant pathogens. Antifungal activity of *F. deltoidea* methanol extract against test pathogens *in vitro* suggests presence of one or more antifungal secondary metabolites in the leaves of this plant. Nevertheless, further investigation is needed to identify the bioactive compounds of *F. deltoidea* leaves to be used as biocontrol fungicide against *G. boninense* and *R. solani*.

The methanol extracts of *P. macrocarpa* were completely effective against *Foc*R4, *G. boninense*, and *R. solani* at all concentrations above 5% (Figure 6). Nonetheless, the concentration of 5% was sufficient to inhibit the mycelial growth of *G. boninense* (58.3%) as compared to *Foc*R4 (PIDG 0%) and *R. solani* (PIDG 32.2%). According to Altaf et al. (2013), variations in the chemical components of *P. macrocarpa* extract greatly affect the antifungal activities of this plant. For example, the growth

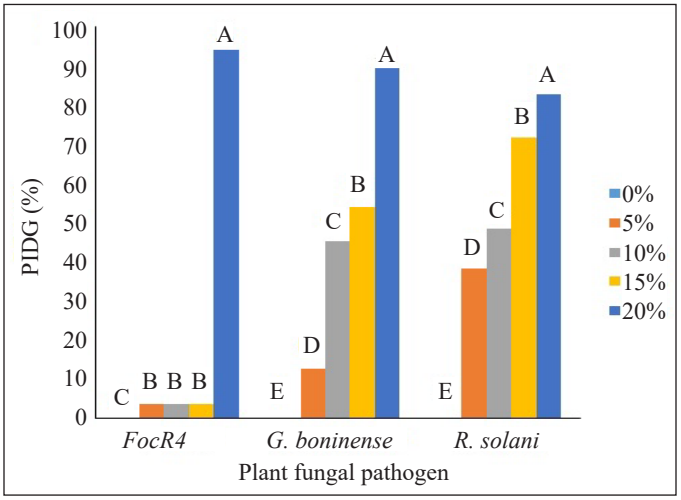


*Figure 4*. Percentage of inhibition of diameter growth of *Piper betle* against selected plant fungal pathogens. Measurement made at 12 days after inoculation (DAI) *Fusarium oxysporum* f. sp. *cubense* R4 (*Foc*R4), 12 DAI for *Ganoderma boninense* and 4 DAI for *Rhizoctonia solani* (R). Values are the means of 5 replicates. Means with the same letter are not significantly different at P = 0.05

inhibition of *Aspergillus niger*, *Fusarium oxysporum*, *Ganoderma lucidum*, and *Mucor indicus* by phorbolesters in *P. macrocarpa* seeds has been reported (Altaf et al., 2013). Furthermore, flavanoids have been identified as the compound accountable for the antifungal activities in higher plants (Cordell et al., 2001). Thus, identification of the bioactive constituents of *P. macrocarpa* leaves is necessary to develop a bio-fungicide.

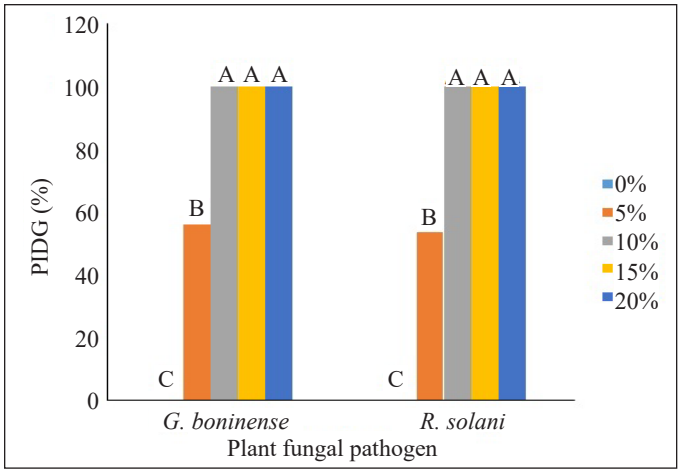


*Figure 5*. Percentage of inhibition of diameter growth of *Ficus deltoidea* against selected plant fungal pathogens. Measurement made at 7 days after inoculation (DAI) for *Ganoderma boninense* and 5 DAI for *Rhizoctonia solani*. Values are the means of 3 replicates. Means with the same letter are not significantly different at P = 0.05

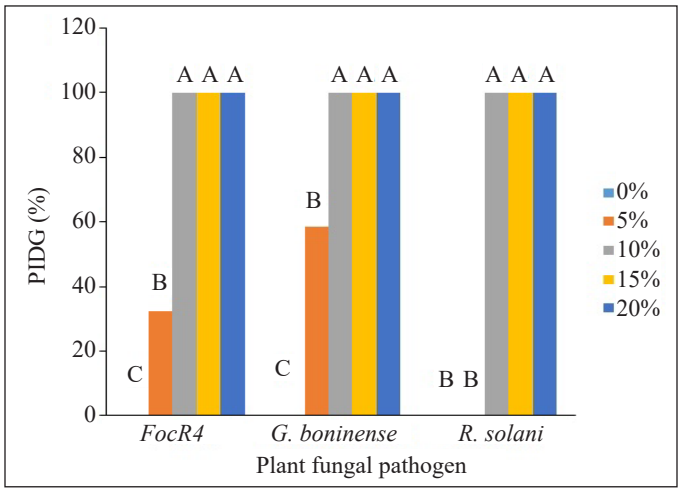


*Figure 6*. Percentage of inhibition of diameter growth of *Phaleria macrocarpa* against selected plant fungal pathogens. Measurement made at 9 days after inoculation (DAI) for *Fusarium oxysporum* f. sp. *cubense* R4 (*Foc*R4), 11 DAI for *Ganoderma boninense*, and 5 DAI for *Rhizoctonia solani* (R). Values are the means of 6 replicates. Means with the same letter are not significantly different at P = 0.05

## CONCLUSION

In this study, *Backhousia citriodora*, *Clinacanthus nutans*, *Ficus deltoidea*, *Phaleria macrocarpa*, and *Piper betle* extracts exhibited different antifungal potential against three economically-important fungal pathogens when tested *in vitro*. Among them, *P. macrocarpa* has been identified as the most effective candidate for development of biofungicide primarily for *Foc*R4, *G. boninense*, and *R. solani* followed by *P. betle*. On the other hand, leaf extracts of *B. citriodora* and *C. nutans* can be used in the management of *Fusarium* wilt disease and soil-borne diseases caused by *R. solani*, respectively. Further experimental investigation into bioactive compounds of these herbal plants as well as trials in both glasshouse and field are strongly recommended for development of novel biofungicides.

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*Review article*

# The Role of Soil Clays in Mitigating or Exacerbating Impacts to Fertility in Crude Oil-contaminated Sites

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## ABSTRACT

About two-thirds of crude oil is produced in countries with tropical and subtropical climates. Many sites in these regions have been threatened by oil spills that can adversely affect soil physical, chemical and biological properties. In some tropical countries, such as Mexico, Venezuela, India, and Nigeria, studies have been conducted to evaluate the effects of petroleum spills on soil fertility, often by monitoring pasture germination or contaminant toxicity. It has been observed that most common impacts to petroleum-contaminated soil occur by two mechanisms: a) by the formation of a thin layer of hydrocarbons on soil particles that results in a reduction in field capacity and causes soil water repellency; and b) by the formation of macro-aggregates (agglomeration) of fine soil particles into coarse particles, thus causing compaction and reduced porosity in the soil. In these studies, it appears that the type and quantity of soil clays influence how severe these impacts may be, being mitigated in the presence of higher contents of smectite clays and being more intense in soils with other fine materials (silts, kaolinite clays, Fe/Al oxides). However, these results have been observed as circumstantial evidence in natural soils. To better understand the relationship between petroleum hydrocarbons and soil clays, an artificial soil system is suggested in which the type and amount of soil clay can be controlled.

*Keywords*: Compaction, kaolinite, petroleum, smectite, tropics, toxicity, water-repellency

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## INTRODUCTION

In this article, the interaction between the type and amount of clay in soil, with respect to negative impacts to soil fertility caused by petroleum hydrocarbon contamination was discussed, especially with respect to water repellency, compaction and toxicity, and a strategy for investigating these interactions systematically in an artificial soil system was proposed.

### Petroleum Production in Tropical and Subtropical Regions and Environmental Regulation

The petroleum industry one of the industries that causes considerable impacts to agriculture and livestock raising (Palma-Cruz et al., 2016). According to the International Energy Agency (IEA) (2016), in 2016 almost two-thirds of petroleum was produced in countries with tropical and subtropical climates (Figure 1). These include countries in the tropics with land-based operations and nearby areas with subtropical climates and petroleum production.

In Mexico, as in other parts of the world, environmental regulations are only developed with respect to the concentration of hydrocarbons in the soil, without regard to the types of soil and the potential impacts (Hernández-Valencia & Mager, 2003; Louisiana Department of Nature Resources [LDNR], 1986; McMillen et al., 2002; Michelsen & Petito Boyce, 1993; Secretaria de Medio Ambiente y Recursos Naturales [SEMARNAT], 2013). This focus on hydrocarbon concentration exclusively, is

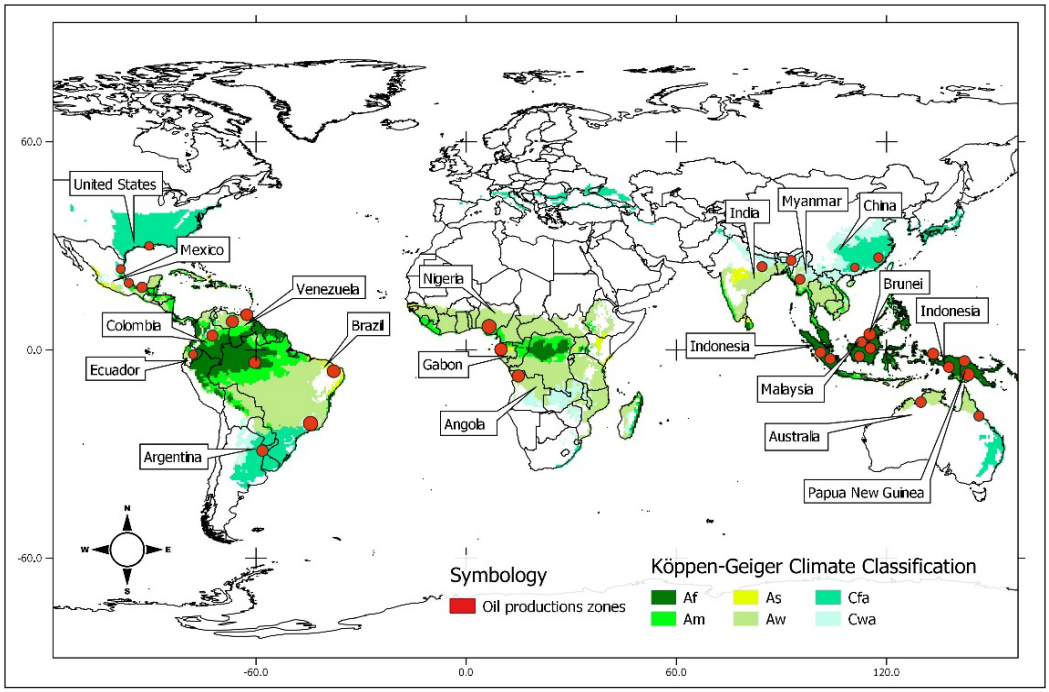



*Figure 1*. Petroleum producing areas in tropical and subtropical regions. Adapted from Rubel and Kottex (2010) using QGIS ver. 3.8. Open code free access to geographical information system (GIS)

based on the supposition that the primary impacts of petroleum in soil are toxicity and potential to leach hydrocarbons to groundwater. However, it has been shown that there are other impacts to soil fertility caused by petroleum hydrocarbons that affect the ability of the soil to maintain a vegetative cover and to be agriculturally productive (Adams et al., 2015; Guzmán-Osorio & Adams, 2015; Marín-García et al., 2015). It is worth mentioning that the personnel in environmental agencies are generally ignorant of the behavior of hydrocarbons in the soil. Thus, essential criteria for the conservation of fertility in petroleum contaminated soils have been overlooked when proposing methods for evaluating site contamination and the effectiveness of remediation projects.

## Effects of Petroleum on Soil Surfaces and Significance for Fertility in Tropical Regions

Certain molecules in petroleum are of very low toxicity, but cause changes in the chemical and physical properties of the soil. They are more prevalent in heavy crude or old spills with weathered oil (Adams et al., 2008a). These contaminants cover soil particle surfaces, interfering in the normal soil-water-plant relationship, causing water repellency and reduced moisture content at field capacity.

Tropical regions have intense sunlight, high temperatures and humid climates, favorable for the natural processes involved in petroleum weathering–volatilization, photolysis, partial biodegradation, chemical oxidation/condensation, and sequestration in soil clays and organic material. Thus, in the tropics these kinds of hydrocarbon molecules are most likely to be produced, have the greatest effect on soil surfaces and fertility, and negatively impact agriculture and cattle-raising (Adams et al., 2008a; Ndimele et al., 2018).

In Mexico, Venezuela, India, and Nigeria research has been carried out to evaluate the effects of petroleum on soil fertility using a grass seed-germination bioassay or contaminant toxicity assay (Barua et al., 2011; Hernández-Valencia et al., 2017; Osuji & Nwoye, 2007; Oyedeji et al., 2012; Vázquez-Luna et al., 2010). This helps in understanding the behavior of these contaminants in soil, and may be an important conceptual tool for decision making concerning the remediation processes of contaminated sites in the tropics. The magnitude of the impacts depends on not only the type and concentration of the oil spilled (Ataikiru & Okerentugba, 2018; Ndimele et al., 2018), but also the kind of soil (De Silva & van Gestel, 2009; Marín-García et al., 2015) (Table 1).

Soil types presented in Table 1 can also be found in other tropical petroleum-producing areas such as the Faja del Orinoco in Venezuela (Hernández-Valencia et al., 2017); in the Niger River Delta in Nigeria (Osuji & Nwoye, 2007); in the upper Assam region in India (Barua et al., 2011); in the southern part of Sumatra (Indonesia) (Yudono et al., 2010); and in regions of Borneo and Papua.

Table 1
*Common soils contaminated in the petroleum-producing zone of Southeastern Mexico*

| Soil type | | Description | Clay | |
|---|---|---|---|---|
| USDA | WRB | | % | Type |
| Psamment | Arenosol | Coarse texture, high permeability, low nutrient storage capacity. Found principally in coastal zones (dunes). | Very low (< 3) | NA |
| Fluvent | Fluvisol | Deep soils with good permeability, medium texture and poor soil horizon development, good superficial drainage, high in nutrients and organic material. Found principally in natural river levees. | Medium (~40 %) | 2:1 |
| Vertisol | Vertisol | High concentration of expandable clays. Principally in tropical and subtropical climates. Vegetation is predominately pasture for cattle and/or forest. | High (~60-65%) | 2:1 |
| Aquent | Gleysol | Saturated with water for the major part of the year, shallow water table. From low-lying areas in alluvial plains. High organic matter and nutrients. | High (~50-55%) | 2:1 |
| Ultisol | Acrisol | Strongly acidic, degraded soils, low base saturation at depth, high concentration of low reactivity clays. Found principally in humid tropical and subtropical areas. | High (~40-50%) | 1:1 |

*Note*. USDA = United States Department of Agriculture (2014), WRB = World Reference Base for Soil Resource (2014)

It is a key to consider that petroleum-contaminated soils are affected principally by a) by the production of thin hydrocarbon laminates on soil surfaces, that produce reduced field capacity and water repellency; and b) by the formation of macro-aggregates, from the agglomeration of smaller soil particles, affecting porosity and compaction (Figure 2). The importance of soil clays on these processes are discussed in the following sections.

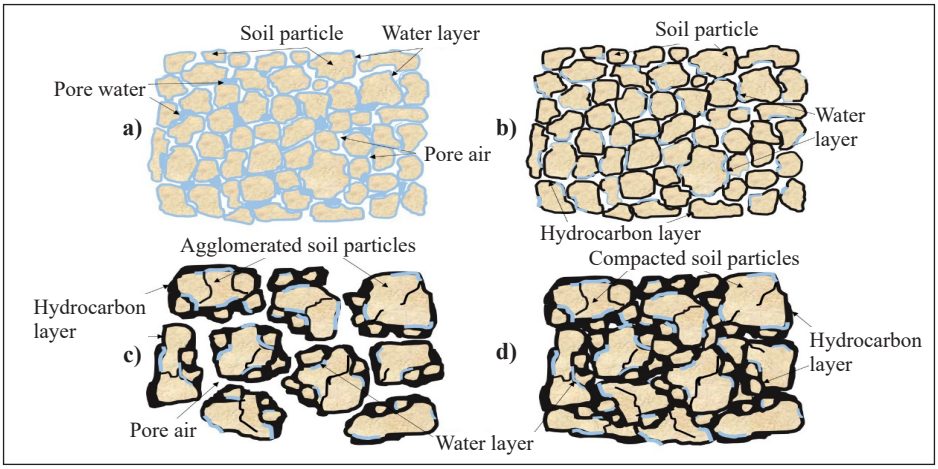



*Figure 2*. Representation of crude oil impacts to soil: a) clean soil at field capacity; b) contaminated soil-formation of hydrocarbon laminates on particle surfaces; c) formation of macro-aggregates – agglomeration of fine particles; d) compaction of contaminated soil-union of macro-aggregates due to an external force

## Importance of Clays in Soil Water Repellency (Production of Crude Oil Laminates)

Soil water repellency prevents soil from moistening (principally at the start of the rainy season), and interrupts the free flow of water through the soil, affecting field capacity and moisture content. The principal cause of repellency is the accumulation of hydrophobic substances on particle surfaces (Hajabbasi, 2016; Jaramillo, 2006). Principally, products resulting from the partial decomposition of vegetable matter (Doerr et al., 2000); due to fires (DeBano, 2000; Dekker & Ritsema, 2000); as well as the contamination from petroleum (Marín-García et al., 2015; Roy & McGill, 1998).

Fertility loss in petroleum contaminated soil begins when hydrocarbons cover soil surfaces (Akinwumi et al., 2014; Litvina et al., 2003; Osuji & Nwoye, 2007; Walter & Omasirichi, 2015). These form a thin layer of hydrocarbons that generate water repellency and reduce the soils' ability to be moistened (Adams et al., 2008b). The normal soil-water relationship is altered and plant productivity affected by the obstruction of roots due to the hydrocarbon layer (Barua et al., 2011; Hernández-Valencia et al., 2017; Li et al., 1997; Quiñones-Aguilar et al., 2003; Figure 2b).

Litvina et al. (2003) developed a conceptual model for this phenomenon in petroleum-contaminated soil (Figure 3). The hydrocarbon layer comprises several components. The mineral component of the soil is overlain and to some extent intermixed with the natural organic (plant-derived) components in the soil, by the interaction between the negative charges in soil clays and the positive charges in the soil organic matter (SOM). The SOM is then overlain with partially degraded petroleum hydrocarbons. During the chemical or biodegradation of the hydrocarbons, polar functional groups are produced, such as alcohols, ketones, aldehydes and carboxylic acids. These groups then interact by hydrogen type bonds with similar functional groups in the SOM. The non-polar parts of

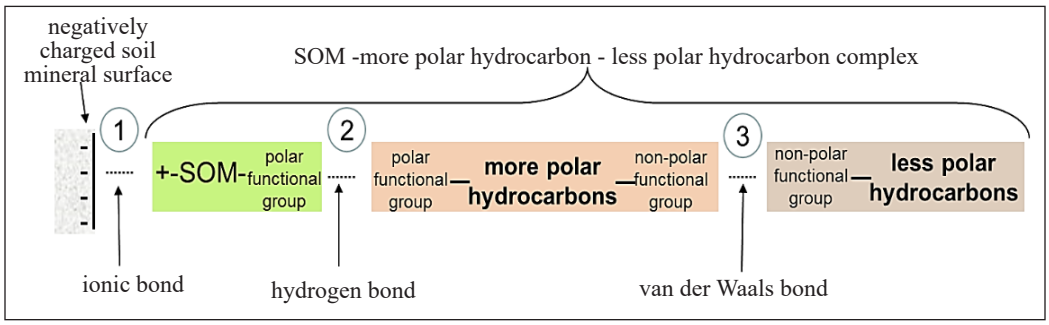



*Figure 3*. Interaction between soil and petroleum: the soil organic matter – hydrocarbon complex. Developed from Domínguez-Rodríguez and Adams (2011). SOM: Soil Organic Matter. 1) Negative charges in clays are attracted to cationic groups in the SOM. 2) Polar groups in the SOM are attracted by hydrogen-type bonds to polar groups in partially oxidized hydrocarbons. 3) The non-polar functional groups in the partially oxidized hydrocarbons are attracted to most hydrocarbons in the mixture, which do not have oxidized groups, by van de Waals type bonds

the partially biodegraded hydrocarbons are then free to interact with the vast majority of non-polar hydrocarbons in the mixture of contaminating oil – by van der Waal forces.

In this model, soils contaminated with hydrocarbon mixtures rich in polar functional groups (such as very heavy crude or weathered oil), would be more apt to from extensive hydrocarbon laminates on the soil surfaces and present more severe water repellency. This is because these kinds of hydrocarbon mixtures have more of the "bridging" compounds that bind the non-polar hydrocarbons to the SOM more effectively. This tendency was confirmed by Morales-Bautista et al. (2016). In agreement with this, the SOM-hydrocarbon complex can be de-stabilized by applying solutions with soluble cations that displace the complex from soil surfaces and thereby reduce or eliminate water repellency. This was confirmed by Alejandro Álvarez (2015) and Contreras-Pérez (2017). These researchers used solutions of sodium hydroxide to restore wettability to oil-contaminated soil, reducing water repellency by up to 75%.

Another reason for soil water repellency mentioned in the literature is forest fires (Ferreira et al., 2005). With more intense fires, soil water repellency becomes more severe and spatially uniform. In contaminated soils, water repellency is more common after catastrophic spills and fires or extended dry periods (Adams et.al. 2008a; Roy & McGill, 1998). This appears to be related to the production of more polar hydrocarbons from the fire itself, and/or the elimination of water from the soil due to evaporation, and the subsequent and intense binding between hydrocarbons and soil surfaces (without an intervening water layer impeding this binding).

Adams et al (2008a, 2015) also found that one of the consequences of soil water repellency was a reduction in field capacity. The hydrocarbons interfered in the interaction between soil particles (especially clays) and water due to the formation of a thin layer of the contaminant on the surfaces, thus reducing the ability to retain water. They also found a negative and lineal correlation between contaminant concentration and field capacity.

Likewise, other authors (Ávila Acosta, 2011; de la Cruz Morales, 2014; Marín García, 2012; Montero Vélez, 2016; Morales Bautista, 2014) have studied the impacts of diverse types of petroleum in different types of soil (Table 2). Among the impacts found, water repellency was common and was strongly associated with the type of petroleum as well as soil type, principally the type and amount of clay in the soil (Morales Bautista, 2014).

Although water repellency is more common in sandy soils (Roy & McGill, 1998), it can also occur in clayey soils. Marín García (2012) found water repellency in a Vertisol contaminated with light, medium and heavy crude (1-8%) and the repellency could be calculated according to the concentration and the type of oil (°API, also known as API degrees or API gravity - a classification system of the American Petroleum Institute). With light

crude, the effects were very moderate (only slight repellency at 8%). However, with heavy petroleum, a strong repellency was found, even at a lower concentration (2%). The heavier crudes, contained more hydrocarbons with polar functional groups serving as chemical "bridges" between the SOM (which also had charged/polar functional groups) and the majority of (non-polar) hydrocarbons in the petroleum, thus enabling more a complete covering of the soil surfaces and more severe water repellency.

The clays, most prevalent in this soil (Vertisol), are smectites, with high shrink-swell capacity. The degree of water repellency was less in this soil than in a medium-textured soil with the same kind of clays (Fluvent) (Morales Bautista, 2014). Thus, with increasing amounts of smectite clays, the intensity of water repellency is reduced. A possible explanation: as the smectites shrink and swell, the thin layer of hydrocarbons on soil particles is ruptured, permitting more contact between the (wettable) mineral surfaces of the soil and water.

With respect to clayey soils with kaolinite, de la Cruz Morales (2014) found that in an Ultisol, the severity of water repellency increased with higher concentrations of crude, and with heavier crudes, coinciding with results reported by Ávila Acosta (2011) and Marín García (2012). Although the kind of clay predominant in this kind of soil (kaolinite) is not expandable and therefore generally has less surface area than in Vertisols, they have more surface area than in sandy soils (Psamments), and tend to present water

Table 2
*Examples of studies related to the effects of petroleum on soil fertility*

| Soil | Petroleum | Concen-tration | Objetive | Scale | Reference |
|---|---|---|---|---|---|
| Psamment (Arenosol) | Light, Medium, Heavy and Extraheavy | 1, 2, 4, and 8% | Evaluate petroleum type and concentration on soil fertility/ toxicity | Labor-atory | Ávila Acosta, 2011 |
| Vertisol | Light, Medium, Heavy and Extraheavy | 1, 2, 4, and 8% | Evaluate petroleum type and concentration on soil fertility/ toxicity | Labor-atory | Marín García, 2012 |
| Psamment (Arenosol), Aquent (Gleysol) and Fluvent (Fluvisol) | Medium, and Heavy | 0.25, 0.5, 1, 2, 4, and 8% | Evaluate petroleum weathering on soil properties | Experimen-tal patio (open air) | Ávila Acosta, 2014 |
| Vertisol, Aquent (Gleysol) and Fluvent (Fluvisol) | Light, Medium, Heavy and Extraheavy | 1, 2, 4, and 8% | Evaluate the type of petroleum (°API) vs. negative effects on soil fertility | Laboratory | Morales Bautista, 2014 |
| Ultisol (Acrisol) | Light, Medium, Heavy and Extraheavy | 1, 2, 4, and 8% | Evaluate petroleum type and concentration on soil fertility/ toxicity | Laboratory | de la Cruz Morales, 2014 |

repellency in an intermediate range. With light crude, the water repellency went from slight (1% oil) to severe (4% and 8% oil). With medium crude, at only 1% oil there was already severe water repellency and with heavy crude at 1% oil there was very severe water repellency. In general, there has been greater water repellency with heavier crudes than with lighter crudes and more water repellency at high concentrations of oil, but the degree of water repellency also depends upon the type and amount of clay in the soil (Table 3).

Water repellency is presented in its two most common manifestations: persistence and severity. Persistence refers to how long it takes a drop of water to penetrate dry soil. It is measured in seconds and reported as the Water Drop Penetration Time (WDPT).

Severity refers to how much surfactant is needed to overcome the water repellency, using ethanol as a week surfactant. The Molarity Ethanol Drop (MED) value refers the concentration (molarity) of ethanol in water that permits a drop of solution to penetrate the soil in ≤10s. In general, soils with no or low clay contents (for example Psamments) have very little surface area, which is completely covered with oil even at low hydrocarbon concentrations, showing high persistence (long times for water penetration). As in Table 3, the Psamment showed extreme persistence and a severity classified as very severe. However, under more moderate hydrocarbon concentrations, this persistence is easily overcome by relatively low concentrations of surfactants, since very little surface area is involved (Adams et al., 2008b). Conversely, soils with high clay contents (such as Vertisols or clayey Aquents) tend to show much lower persistence, but once affected, much higher

Table 3
*Concept table on the degree of water repellency (WDPT and MED) with different types and quantities of clay*

| Crude oil | Type of Water Repellency | Quantity of clay and soil type | | | |
|---|---|---|---|---|---|
| | | Very low (Psamment/ Arenosol)* | Medium (Fluvent/ Fluvisol)** | High (Vertisol, Aquent/ Gleysol)*** | High (Ultisol/ Acrisol)**** |
| Light | WDPT | Extreme[a] | Null[a] | Null[a] | Extreme[a] |
| | MED | Very severe[b] | Low[b] | Null[b] | Very severe[b] |
| Medium | WDPT | Extreme[a] | Slight to strong[a] | Slight[a] | Strong to extreme[a] |
| | MED | Very severe[b] | Low to severe[b] | Low to very severe[b] | Low to very severe[b] |
| Heavy | WDPT | Extreme[a] | Slight to strong[a] | Slight to strong[a] | Extreme[a] |
| | MED | Very severe[b] | Low to very severe[b] | Very severe[b] | Very severe[b] |
| Extra-heavy | WDPT | Extreme[a] | Null to strong[a] | Null to strong[a] | Severe[a] |
| | MED | Very severe[b] | Low to very severe[b] | Null to Very severe[b] | Very severe[b] |

*Note*. *Ávila Acosta (2011), **Ávila Acosta (2014) and Morales Bautista (2014), ***Marín García (2012) and Morales Bautista (2014); ****de la Cruz Morales (2014), WDPT = water repellency persistence, MED = water repellency severity, a = water repellency persistence, b = water repellency severity

severity. This is especially pronounced in soils with 2:1 clays (expandable) due to the internal surface areas available in addition to the external surfaces (such as in Vertisols, Aquents, and to a lesser degree, Fluvents). Meanwhile, in clayey soils that are not expandable (in Ultisols, for example, with high kaolinite contents), the lack of internal surfaces in the clays appear to reduce the ability of the soil to overcome water repellency. Although not as repellent as soils without clays (Psamments), in comparison to alluvial soils, they are much more likely to develop both persistence and severity.

In a comparison between alluvial soils (Fluvent, a Vertisol and an Aquent), Morales Bautista (2014) found that the Vertisol and Aquent showed much less impact from petroleum to soil water repellency. The proposed reason was the shrink-swell capacity of the 2:1 clay being more abundant in the Vertisol and the Aquent. The frequent shrinking and swelling in these soils were postulated to break apart hydrocarbon laminates and expose hydrophilic soil surfaces, thus reducing water repellency. Therefore, soils with higher amounts of smectite clays tend to show less water repellency, congruent with that observed by Marín García (2012).

In summary, soils without clays (Psamments) are most vulnerable, followed by soils basically without 2:1 clays (Ultisols), while soils with moderate amounts of 2:1 clays (Fluvents) tend to have moderate water repellency when contaminated, and soils with abundant 2:1 clays (Vertisols and clayey Aquents) tend to have little to no water repellency when contaminated by petroleum hydrocarbons.

## Compaction (Particle Agglomeration) in Clayey Soils

Hydrocarbons also result in the formation of aggregates caused by the viscosity of hydrocarbons adhered to particle surfaces (Marín García, 2012). Adams et al. (2008b) explained how the chemical structures of residual hydrocarbons might have "sticky" terminals that act as agglomeration agents, uniting fine particles (clays) into larger (sand-sized) particles, causing soil compaction. During petroleum weathering, alcohols, keto-groups, aldehides and carboxylic acids, are produced, similar to the kinds of functional groups common in asphaltenes. These give the resulting hydrocarbon mixture strong binding characteristics. According to Montero Vélez (2016), contamination with petroleum mixtures with these kinds of compounds results in permeability changes and percolation of water through the soil (Figures 2c and 2d). A conceptual model of this is shown in Figure 4. As shown, the partially oxidized hydrocarbons play a crucial role in both the formation of hydrocarbon laminates and agglomeration of small particles into larger clusters.

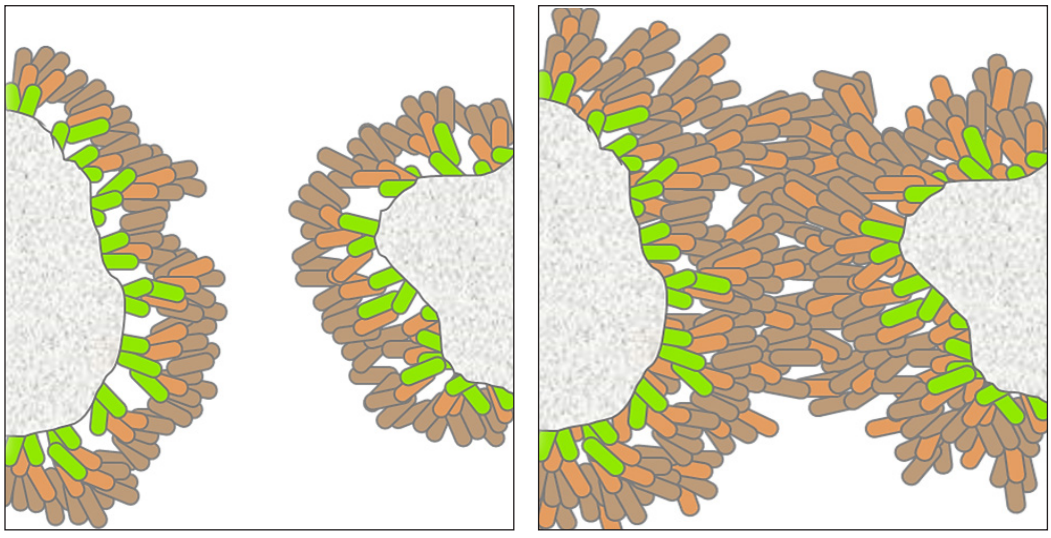
Compaction results in increased bulk density and reduced pore spaces, reducing the movement of water and air, and limiting biological activities (Barik et al., 2011; Hajabbasi, 2016; Nawaz et al., 2013). In agricultural soils, compaction is caused by the constant movement of heavy machinery

(Adams et al., 2008b; Palma-López et al., 2007), or by intensive hoof pounding by cattle.

Nonetheless, in soils with petroleum contamination, this problem intensifies. This is due to the sticky ends in asphaltenes, resins and polar compounds found in weathered oil that increases the viscosity of the oil and its adherence to soil, thus generating agglomeration between soil particles (Adams et al., 2008a, 2008b, 2015).

Nawaz et al. (2013) mentioned compaction as physical soil degradation causing changes in soil structure and the soil-water-air dynamic, as well as reduced plant growth from less root penetration, lowering crop production (Adams et al., 2015; Batey, 2009; Suuster, 2011; Trujillo-Narcía et al., 2012). Likewise, Adams et al. (2015), found a 56% reduction in biomass production in petroleum-contaminated soil. This was attributed to compaction from the oil caused by the more polar compounds found in the petroleum hydrocarbon mixture (Batey, 2009).

Others, such as de la Cruz Morales (2014), Morales Bautista (2014), and Montero Vélez et al. (2016) found a tendency in petroleum-contaminated soils where the degree of the negative compaction impacts depended not only on the type of oil (light, medium and heavy) and its concentration, but also the type of soil, principally to the type and quantity of clays. Nawaz et al. (2013) showed that soil compaction varied based on the type of soil, principally, soil particle size. For example, soils with



(a) (b)

*Figure 4*. Formation of hydrocarbon laminates on soil surfaces and particle agglomeration by petroleum. From the Litvina et al. (2003) conceptual model. a) formation of laminates and water repellency; b) agglomeration of small particles into larger clusters. Partially oxidized hydrocarbons are key to these interactions, forming chemical bridges between the soil organic matter and the mass of non-oxidized hydrocarbons, thus facilitating the laminates formation even at low concentrations (a). Also, due to their asphaltene-like nature, they are strong binders of particles into larger agglomerations at higher concentrations (b)

kaolinites (for example Ultisols) tend to compact naturally, while soils with smectites (Vertisols, clayey Aquents, and to a lesser extent Fluvents) tend to expand (constant shrink-swell). Due to these different intrinsic properties of soil clays, industrially they find different uses. Kaolinites are used for the production of ceramics and bricks, while smectites are employed as agglomerating agents for heat resistant materials (Díaz Rodríguez & Torrecillas, 2002) and in the petroleum industry, for oil well sealers.

As mentioned, compaction problems can be more be severe with heavier crudes. de la Cruz Morales (2014) observed that in an Ultisol (1:1 clays), compaction problems seemed greater with heavier crudes, suggesting that the type and concentration of petroleum also plays an important role. Although potentially almost all soils could suffer compaction, in contaminated soils it appears to be much more problematic in Ultisols contaminated with heavy crude or weathered oil.

**Toxicity in Clayey Soils**

Some researchers (Ávila Acosta, 2011; de la Cruz Morales, 2014; Marín García, 2012; Montero Vélez, 2016; Morales Bautista, 2014) have found that the type of soil plays a key role in the toxic impact of chemical compounds spilled in soil. Petroleum is a complex mixture of compounds mostly containing hydrogen and carbon, but also small amounts of nitrogen, sulfur, oxygen and some metals. The hydrocarbons cause alterations in soil by several mechanisms: direct toxicity to soil organisms, reduction in soil humidity/nutrient availability, and changes to pH and salinity (Adams et al., 2008b). The toxicity varies according to the type of petroleum. The lighter hydrocarbon fractions (with lower molecular weight) are more toxic, while the heavier fractions (with higher molecular weight) present less toxicity (Adams et al., 2008b; Tang et al., 2011).

For example, Ávila Acosta (2011) found that in a Psamment, the toxicity of four kinds of petroleum increased with concentration. The most toxic contaminant was light crude at 2-8%, followed by medium crude at 8%, while heavy crude did not show toxicity even at the 8% level. Thus, the lighter fractions cause greater toxicity, and this diminishes as the oil type becomes heavier. However, Marín García (2012) found that in a Vertisol, light, medium, heavy and extra-heavy crudes did not produce toxicity at concentrations of 1-8%. These kinds of soils with 2:1 clays (smectites) have a much greater surface area and large shrink-swell capacity that provide for a higher adsorption. Likely, this characteristic mitigated toxicity, since the petroleum hydrocarbons were adsorbed onto the soil clays and therefore, less bioavailable. In contrast, Ávila Acosta (2011) found that in a Psamment, a coarse-textured soil with very low clay content (thus low surface area), much higher toxicities were found.

Likewise, de la Cruz Morales (2014) found that in an Ultisol, only light crude caused toxicity in concentrations of 2-8%, while the medium, heavy and extra-heavy crudes did not cause toxicity. In this kind

of soil, the clays are not expandable (type 1:1), with a limited surface area. However, they do have greater surface area than sandy soils (like Psamments). This may help adsorb contaminants and thus show lower toxicity than that found by Ávila Acosta (2011). In these studies tendencies are observed: toxicity depends on the type of soil, with soils with very low quantities of clays (Psamment) showing the highest toxicity, kaolinite-rich soils (Ultisol) with intermediate toxicities, and smectite-rich soils (Vertisol) with relatively low toxicities.

Lastly, Morales Bautista (2014) found that among alluvial soils (Fluvent, Vertisol, and Aquent), the greatest toxicity was observed in the Fluvent contaminated with light and medium petroleum. At only 1% of light or medium crude, the Fluvent showed considerable toxicity, while the Vertisol and the Aquent did not show toxicity at this level. This is congruent with that reported by Marín García (2012) for a Vertisol. Likewise, Morales Bautista (2014) found that with increasing clay content, the toxicity decreased, as also found by Ávila Acosta (2011), Marín García (2012), and de la Cruz Morales (2014). These authors observed a tendency in which the soil texture played in important part in toxicity, such that among the alluvial soils, the most affected by toxicity was the soil with the least amount of clay, in contrast to the Vertisol and Aquent (with a high clay content).

**Plant Yields in Clayey Soils**

Although fertility is one of the soil qualities that leads to the necessary conditions for plant development, this may be strongly affected by physical-chemical changes in soil. One of the principal causes is oil spills, which reduce plant yields. This effect was shown by Montero Vélez (2016), who observed a reduction in plant yield of Humidicola grass (*Brachiaria humidicola*) in several soils: Psamment, Fluvent, Vertisol, Aquent, and Ultisol, all contaminated with 1% of heavy crude. In these soils, reductions in pasture production were found to be 22%, 51%, 23%, 10%, and 67%, respectively.

Montero Vélez (2016) considered that the relative amount of reduction in plant yield was associated with the following: 1) the impact is relatively low in sandy soil (Psamment), basically without clay and few problems from compaction; 2) relatively low impacts in soils with large amounts of smectites (Vertisol, Aquent), in which the shrink-swell properties rupture hydrocarbon laminates that may form on the soil (thus mitigating problems from water repellency and compaction); and 3) relatively high impacts in soils with large amounts of fine particles other than smectites; silts in the Fluvent and kaolinte (+amorphous Fe/Al oxides) in the Ultisol.

In the last few years, other researchers have also carried out studies on the effect of petroleum on fertility in pastures. For example, Vázquez-Luna et al. (2010) found that high hydrocarbon concentrations damaged plants and reduced growth due to the hydrocarbons covering plant roots and interfering in nutrient uptake. Likewise, Hernández-Valencia et al. (2017) observed impacts in the germination of pastures

(*Megathyrsus maximus* and *Urochloa brizantha*) that was related to the type and concentration of petroleum. With higher °API (lighter oils), and higher concentrations, reduction in germination was more severe, due to the higher content of aromatics and saturates in the oil (more toxicity). Conversely, Barua et al. (2011), mentioned the inhibition of several herbaceous species (*Axonopus compressus*, *Cynodon dactylon*, *Cyperus brevifolius*, and *Eclipta prostrate*), and considered that it was due to: 1) reduced pore space in the soil that makes gas exchange more difficult; and 2) to the hydrophobic properties of petroleum covering seeds, acting as a physical barrier, reducing oxygen and water availability, and reducing gas interchange. This is in agreement with Osuji and Nwoye (2007): the partial covering of soil particles with hydrophobic hydrocarbons could reduce the soil water retention capacity (field capacity) due to a significant reduction in clay bonding.

Alternatively, Morales-Bautista et al. (2016) indicated that the relative amount of polar functional groups in the petroleum caused compaction-agglomeration, reduction in field capacity, reduction in cationic exchange capacity and the formation of soil water repellency. These functional groups are present principally in heavy and extra-heavy crude. These findings are in agreement with Adams et al. (2015) and Montero Vélez (2016), who observed a larger reduction in pasture biomass (*Brachiaria humidicola*) with increasing concentrations of extra-heavy crude. This could be from soil compaction interfering with root penetration, reducing the capacity of the pasture to obtain sufficient moisture and nutrients. Likewise, Oyedeji et al. (2012) mentioned the reduction in plant height and thickness (*Abelmoschus esculentus*) in contaminated soil that could be due to the lack of available water, and thus the mobility and absorption of plant nutrients.

Méndez-Natera et al. (2007) found that petroleum covered root surfaces in a thin layer, altering the absorption of water and nutrients, and reducing plant growth (reduction in respiration and photosynthesis rates). In this sense, some research confirms that the light fraction of petroleum (naphtha) is 20 times more toxic than the heavy fraction (Chaineau et al., 1997). However, Ferrera-Cerrato (1995) mentioned that some plant species could grow in hydrocarbon contaminated soils, and furthermore, actively contributed to hydrocarbon degradation in the rhizosphere. Likewise, Zavala-Cruz et al. (2005) mentioned that pastures had phytoremediation potential. Their adaptation to petroleum-contaminated soil was able to reduce the total petroleum hydrocarbon (TPH) content by 48% after 3.5 months of cultivation with Humidicola grass (*Brachiaria humidicola*) in two soils, an anthropic Entisol and an Ultisol, with Aleman (German) grass (*Echinochloa polystachya* (H. B. K.) Hitchcok) in an Aquent, and with Egyptian grass (*Brachiaria mutica* (Forksskal) Stapf). Thus, measurement of plant growth in contaminated soils may provide better knowledge of the degree of soil fertility impacts.

**Artificial Soil for Systematic Investigation of Soil Clay - Petroleum Interactions (Strategy)**

Many research gaps exist in our understanding of how clays influence soil fertility in petroleum-contaminated soils. No current information can thoroughly explain how the type and concentration of clays affect plant yields in petroleum-contaminated soils. Although previous studies showed a tendency in the behavior of water repellency, compaction, toxicity and plant yield concerning the type and quantity of soil clays, these were observed in natural soils. By studying natural soils, a more precise evaluation is made of the type and magnitude of the impacts caused by petroleum in regional soils of interest. None-the-less, these are limited by the type and quantity of clays in naturally occurring soils in a particular region. To date, there has been no systematic study of how the kind and amount of clay affect common problems in petroleum-contaminated soils. The studies on natural soils do not allowing for a systematic evaluation, where the type and quantity or soil clay can be varied, to determine with greater certitude, how clays influence the mitigation (or exacerbation) of impacts in contaminated soils.

The formation of soil is very complex (Zavala-Cruz et al., 2011), and generates a heterogeneous distribution of organic compounds, minerals, water and gases. This complicates the understanding of the processes that are carried out in the soil and the interactions between components (Guenet et al., 2011). This is because it is impossible to manipulate the proportions of the different components that interact with each other in a natural soil. However, by constructing an artificial soil, one can better understand the interactions in a systematic manner.

To determine how, and in what degree, the type and quantity of soil clay affects water repellency, compaction, toxicity and plant yield with greater certainty and precision, and thus to overcome the research gap, it is necessary to study an artificial soil system. In such a system, the type and quantity of soil clay can be controlled, as well as the quantity of sand and organic matter. In this way, the interactions between the diverse petroleum types and the different types of soil can be better understood. The information generated by such a study could be a useful tool for decision making in remediation projects, and to establish soil restoration criteria and techniques.

On the international level, several authors had conducted studies with artificial soils to systematically study toxicity of different contaminants, and in which at least one soil component was controlled (De Silva & van Gestel, 2009; Shanmugasundaram et al., 2014). Other research had focused on using artificial soils as a tool for analyzing and better understanding biological processes in soil, systematically modifying at least one variable of interest (Ellis, 2004; Guenet et al., 2011). It is worth mentioning that there already exist methods for the preparation of artificial soils, proposed by various agencies, including the American Society for Testing and Materials (ASTM)

(2004), the International Organization for Standardization (ISO) (1993, 1998), the Organization for Economic Co-operation and Development (OECD) (1984), the United State Environmental Protection Agency (USEPA) (1989) that are used for acute and subchronic toxicity bioassays. One advantage of making an artificial soil is that it allows for the creation of an environment with characteristic that are similar to those present in natural soils (Ellis, 2004; Saberi et al., 2018). Furthermore, such systems can be used to evaluate future contamination scenarios such as petroleum spills.

The components of a systematic study to evaluate the interaction between clays and impacts to soil could include a system similar to one of the currently used methods for making artificial soil, for example that proposed by the OECD (Protocol No. 207, OECD 1984). According to the OECD, artificial soil is defined as a mixture of 70% fine quartz sand (50% of the particles between 0.05 – 0.2mm), 20% kaolinite clay and 10% *Sphagnum* moss, finely crushed (Hofman et al., 2009). This artificial soil was developed to be a standardized medium "similar to soil and was introduced as a substrate for acute toxicity test with earthworms (Hofman et al., 2009).

Based on this method, modifications could be included to study the impacts of petroleum in soil, according to the type and quantity of clays, as well as the type and concentration of petroleum (see Table 4).

By systematically varying the amount and type of clay in the artificial soil, as well as the type and concentration of petroleum in the soil, the role that clays play with respect to water repellency, field capacity, toxicity and plant yield can be determined. Some overall tendencies may be found that predict what kinds of soils are most vulnerable, what remediation criteria are most important to restore, and what could be the most effective remediation or restoration techniques. As seen by Montero Vélez (2016), for vulnerable soils (for example Ultisols) the concentrations of heavy crude that do not limit plant growth are very, very

Table 4
*Experimental design for use of artificial soil in the evaluation of clay-petroleum interactions in contaminated soil (proposed)*

| Independent Variables | Constants | Dependent Variables | Method |
|---|---|---|---|
| Type of clay (kaolinite or smectite) | Quantity and type of organic matter (10% *Sphagnum* moss) | Water repellency and field capacity | MED, WDPT, FC (Adams et al., 2008a) |
| Quantity of clay | Type and quantity of sand (percent to complete 100%) | Compaction | Penetrometer (American Society of Agricultural and Biological Engineers [ASABE], 1999) |
| Type of crude petroleum (light, medium, heavy) | | Acute toxicity (earthworm bioassay) | Direct contact modification of OECD (Domínguez-Rodríguez et al., 2020) |
| Concentration of petroleum | | Plant yield | *Brachiaria humidicola* grass biomass (Montero Vélez, 2016) |

low (<100 mg $Kg^{-1}$) and not technically achievable or economically feasible. A better strategy may be to develop techniques to overcome the soil impacts (by adding soil conditioners, for example) rather than reduce the hydrocarbon concentration.

Additionally, the results of this kind of systematic study will need to be compared with real regional soils and validated under field conditions. Such scale-ups may provide additional information to better understand and improve the conceptual or descriptive models developed using the artificial soils.

## CONCLUSION

The negative impacts of crude oil on soil fertility in terms of toxicity, soil water repellency and plant yields depend not only on the type and concentration of oil spilled, but also on the type and quantity of soil clay. There is abundant circumstantial evidence in the published literature on soil compaction and the formation of soil water repellency in petroleum-contaminated soil. In soils with higher contents of expandable clays (smectites), these kinds of impacts tend to be less with fewer problems for soil fertility. These investigations have all been carried out on natural soils; however, there is a need for a systematic study in which the type and amount of soil clay can be experimentally controlled, using an artificial soil system.

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# Nutrient Digestibility, Metabolizable Energy and Carcass Traits of Broilers Fed White and Yellow Cassava Root Meals Supplemented with Different Additives

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## ABSTRACT

Cassava root is rich in energy but low in protein. New varieties developed exhibit better nutritional profile. An 8-week experiment was conducted to investigate the effect of dietary supplementation of 2 varieties of cassava root meal (CRM) with various additives on nutrient digestibility energy, metabolizable and carcass traits of broilers. Two hundred and forty unsexed day-old broilers were allotted to 8 dietary treatments in a 2 x 4 factorial arrangement of white (TME 419) and yellow (ITA/IBD/1368) CRM supplemented with no additive, amino acids (methionine and lysine), enzyme and amino acids + enzyme (A.A + Enz). The experiment lasted for the starter (0 - 4 weeks) and finisher (5-8weeks) phases. Variety effect showed higher ($p<0.05$) nutrient digestibilities in finisher broilers fed with white cassava than yellow. White cassava + amino acids showed higher EED and ASHD while yellow cassava with amino acids + enzyme yielded improved nutrient digestibilities. White cassava variety revealed higher ($p<0.05$) metabolizable energy values than yellow. Broilers fed white cassava + enzyme had the highest ($p<0.05$) metabolizable energy values. In conclusion, supplementing yellow CRM with A.A + Enz improved nutrient digestibility only at the starter phase. Supplementation of white cassava diet with enzyme and amino acid at the starter

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and finisher phases respectively, improved energy metabolizablility.

*Keywords*: Additives, broilers, carcass, cassava, energy metabolizablility, nutrient digestibility

## INTRODUCTION

Cassava (*Manihot esculenta* Crantz) root is a cheap and sustainable energy feedstuff with potential to replace most conventional cereal grains in the tropics (Oso et al., 2014). Cassava root is rich in digestible starch, gross energy content (El-sharkawy, 2012) and has been used to a limited extent in poultry nutrition (Eruvbetine et al., 2003; Oso et al., 2014). However, high fibrous content (of its peel), the presence of hydrocyanide (HCN) residues, reduced protein levels, poor protein quality and reduced concentration of sulphur containing amino acids in cassava root, constitute the major constraints to its maximal utilization as energy feedstuff in poultry nutrition (Banea-Mayambu et al., 1997). During cassava processing which converts cyanide to a less toxic thiocyanate, the enzyme 'rhodanese' contained in cassava root utilizes the constituent methionine and other sulphur containing amino acids as sulfur donor (Cardoso et al., 2005). Thus, sulphur amino acids become grossly deficient in cassava-based diets fed to poultry birds. Hence, to harness the rich energy potential of cassava root maximally in poultry nutrition, it is essential to supplement cassava root-based diets with limiting amino acids. Supplementation of fibrous feed ingredients with feed additives like enzyme improves utilization of the feed and reduces intestinal viscosity, thereby improving gut health and nutrient digestibility (Abdulrashid et al., 2007; Kayode, 2009).

The conventional white cassava roots and products are deficient in β-carotene and other carotenoids (Khajarern & Khajarern, 2007; Omole, 1977) which are needed in poultry diet to prevent *in vivo* oxidative stress with the attendant effects on animal products (Ngiki et al., 2014). Yellow cassava on the other hand contains high levels of β-carotene, which is a precursor to vitamin A. β-carotene will enhance nutritional properties of yellow cassava and this could take care of some of the deficiencies that may be associated with lack of β-carotene and other carotenoids in white cassava. Previous studies on the practical inclusion of whole cassava root meal as energy feedstuff in feed for poultry (Aderemi et al., 2012; Akapo et al., 2014; Kyawt et al., 2015; Oso et al., 2014), have limited information on the use of biofortified (yellow) cassava in the feed of poultry. This study therefore seeks to investigate the effect of synthetic amino acids (methionine and lysine) and cellulase enzyme supplementation of unpeeled white and yellow cassava root meals (WCRM and YCRM respectively) on nutrient digestibility, energy metabolizablility and carcass traits of broiler chicken.

## MATERIALS AND METHODS

### Experimental Location

The experiment was carried out at the Poultry Unit of Ogun-Oshun River Basin Development Authority, Abeokuta (7° 12' 1.0" N, 3° 26' 13.2" E), Nigeria, West

Africa. This is in the tropical sub-savannah region with an average ambient temperature of 32.91°C and a relative humidity of 79.25 per cent. It receives a mean precipitation of 1,685 mm per annum (Ogun-Oshun River Basin Development Authority [OORBDA], 2016).

## Preparation of Cassava Root Meal

Freshly harvested white cassava variety (TME 419) and yellow cassava variety (ITA/IBD/1368) were gotten from the Federal University of Agriculture, Abeokuta farm for the study. Each variety was thoroughly washed with clean water (to be free of dirt and sand) and chipped into smaller pieces as described by Oso et al. (2010). The chipped cassava tubers obtained from each variety were sun-dried separately until they reached a moisture content of approximately 10–12 per cent. The dried chips were collected, bagged, stored, and subsequently milled (2.5-mm sieve) separately to obtain the WCRM and YCRM. The processed cassava root meals were later mixed with other feed ingredients to formulate the experimental diets.

## Chemical Composition of White and Yellow Cassava Root Meals

Samples of the cassava root meals were analysed to determine their chemical constituents using the method described by the Association of Official Analytical Chemists (Horwitz, 2005). The fibre fractions that include the neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) were determined according to the methods of Van Soest et al. (1991). Hemicellulose was calculated as the difference between NDF and ADF, while cellulose was calculated as the difference between ADF and ADL. Also, the cyanide content of the samples was determined following the method of Bradbury et al. (1991). Mineral content Ca, P, Cu, Mg, Mn, and Zn as well as β-carotene of the cassava root meals were determined using Horwitz (2005) and (Association of Official Analytical Chemists [AOAC], 1997), respectively (Table 1).

Table 1
*Chemical composition of white (TME 419) and yellow (ITA/IBD/1368) cassava root meals*

| | White cassava | Yellow cassava |
|---|---|---|
| Proximate components (%) | | |
| Dry matter content | 90.42 | 91.33 |
| Crude protein | 2.20 | 3.56 |
| Ether extract | 0.76 | 0.52 |
| Crude fibre | 1.26 | 1.09 |
| Ash | 2.34 | 2.90 |
| Nitrogen free extract (NFE) | 83.77 | 82.98 |
| β-carotene (µg/100 g) | 15.42 | 349.01 |
| Gross energy (MJ/kg) | 14.80 | 14.46 |
| Hydrocyanide HCN (mg/kg) | 26.60 | 25.40 |
| Fibre fractions (%) | | |
| Nitrogen detergent fibre (NDF) | 26.59 | 24.95 |
| Acid detergent fibre (ADF) | 15.58 | 14.06 |
| Acid detergent lignin (ADL) | 3.37 | 2.53 |
| Hemicellulose | 11.01 | 10.89 |
| Cellulose | 12.21 | 11.53 |
| Mineral content (mg/g) | | |
| Ca | 3.55 | 3.72 |
| P | 0.80 | 0.90 |
| Cu | 0.28 | 0.32 |
| Mg | 0.40 | 0.46 |
| Mn | 0.14 | 0.22 |
| Zn | 0.11 | 0.10 |

**Experimental Birds and Management**

A total of 240-day-old, unsexed broiler chickens of Marshall® strain were distributed at random between 24 pens. Three pens were assigned to each of the eight-dietary treatment in three replications of 10 birds per replicate. The birds were brooded and reared intensively on a deep litter housing system with dried wood shavings as the litter material. Feed and water were offered *ad libitum.* The experiment lasted for eight weeks (0–4 weeks for the starter phase and 5 – 8 weeks for the finisher phase).

**Dietary Treatments**

White and yellow cassava root meals separately replaced 30 per cent of maize as the basal diet in the experimental diets, with different types of additives as follows: there was no additive in one of the diets (control) while the remaining three contained recommended levels of synthetic amino acids (15g methionine and 5g lysine), exogenous enzyme (3g cellulase), and a combination of both amino acids and enzyme. There were eight dietary treatments laid out in a 2 × 4 factorial arrangement, i.e. two varieties of cassava and four types of additives, as indicated below:

- Dietary Treatment 1: WCRM + No additive
- Dietary Treatment 2: WCRM + Synthetic amino acids
- Dietary Treatment 3: WCRM + Exogenous enzyme
- Dietary Treatment 4: WCRM + Synthetic amino acids + Exogenous enzyme
- Dietary Treatment 5: YCRM + No additive
- Dietary Treatment 6: YCRM + Synthetic amino acids
- Dietary Treatment 7: YCRM + Exogenous enzyme
- Dietary Treatment 8: YCRM + Synthetic amino acids + Exogenous enzyme

The composition of the basal diets for both starter and finisher phases are as shown in Table 2.

**Nutrient Digestibility Determination**

At the end of starting and finishing phases, 2 birds from each of the 3 replicates per dietary treatment (48 birds per phase) were selected at random and housed individually in clean-wired floor metabolic cages fitted with individual feed troughs and facility for separate excreta collection. The birds were acclimatized for 2 days prior to the commencement of the 3-day collection period. Birds in each treatment group were fed with the respective test diets and excreta were collected daily for 3 days. The daily feed intake and excreta voided were weighed for the 3-day period. Feed intake was measured by deducting the weight of left-over feed from the weight of feed given daily. The daily excreta voided following the feeding procedure was collected quantitatively and dried at 65°C until moisture content was < 10% and the total collection per group of birds at the expiration of 3-day digestibility trial was pooled and ground. The proximate composition of feed and dried excreta

Table 2
*Gross composition of experimental diets fed to starter and finisher broilers (g/kg)*

| | Starter | | Finisher | |
|---|---|---|---|---|
| Ingredients | WCRM | YCRM | WCRM | YCRM |
| Maize | 320 | 320 | 378 | 320 |
| White cassava | 136 | – | 162 | – |
| Yellow cassava | – | 136 | – | 136 |
| Soya bean meal | 290 | 290 | 204 | 290 |
| Fish meal (70%) | 10 | 10 | – | – |
| Groundnut cake | 90 | 90 | 122 | 122 |
| Wheat offal | 100 | 100 | 80 | 80 |
| Palm oil | 10 | 10 | 20 | 20 |
| Bone meal | 20 | 20 | 17 | 17 |
| Oyster shell | 15 | 15 | 8 | 8 |
| Methionine | 2 | 2 | 2 | 2 |
| Lysine | 1.5 | 1.5 | 1.5 | 1.5 |
| *Premix (broilers) | 2.5 | 2.5 | 2.5 | 2.5 |
| Common salt | 3 | 3 | 3 | 3 |
| *Total* | 1,000 | 1,000 | 1,000 | 1,000 |
| *Determined analyses* | | | | |
| Metabolized energy (kcal/kg) | 2,917.91 | 2,881.44 | 3,092.91 | 3,081.44 |
| Crude protein (%) | 22.90 | 22.97 | 20.03 | 20.11 |
| Crude fibre (%) | 5.56 | 5.54 | 5.77 | 5.89 |
| Ether extract (%) | 5.48 | 5.35 | 5.60 | 5.58 |

*Note.* Each 2.5 kg broiler premix contains 1.25 kg Vitamin Premix (Vit. A 10,000,000 I.U; Vit. D3 2,000,000 I.U; Vit. E 10,000 mg; Vit. K3 2,000 mg; Vit. B2 4,000 mg; Vit. B6 1,500 mg; Vit. B12 10 mg; pantothenic acid 5,000 mg; biotin 20 mg; niacin 15,000 mg; and antioxidant 125,000 mg), 1 1.25 kg Mineral Premix (copper 5,000 mg; iodine 1,200 mg; selenium 200 mg; cobalt 200 mg; iron 20,000 mg; zinc 50,000 mg; manganese 80,000 mg; and choline chloride 200 g

samples was analyzed for dry matter, crude fibre, ether extract, ash, and crude protein (N × 6.25) using standard methods of AOAC (2000).

The feed intake: The feed intake was calculated using the formula:

$$\text{Feed intake per bird (g)} = \frac{\text{Feed supplied(g)} - \text{Left over feed(g)}}{\text{Total number of Birds}}$$

Dry matter digestibility: The dry matter digestibility was calculated using the formula:

$$\text{Dry matter digestibility (\%)} = \frac{\text{Weight of feed intake (g/D M)} - \text{Weight of feed dropping (g/D M)} \times 100}{\text{Weight of feed intake (g/D M)}}$$

From the results of the proximate composition of both the feed and excreta

samples, the digestible crude protein was calculated as:

$$\text{Digestibility Crude Protein (\%)} = \frac{(\text{Weight of feed intake (DM)} \times \%\text{CP in diet}) - (\text{Weight of dropping (DM)} \times \%\text{CP in dropping}) \times 100}{(\text{Weight of feed intake (DM)} \times \%\text{CP in diet})}$$

The same method was used to calculate the digestibility of fat, crude fibre, ash and NFE.

**Apparent and True Metabolizable Energy**

At the end of the digestibility trials at both starter and finisher phases of the experiment, the birds in the metabolic cages were starved of diets and given unrestricted access to clean water for 24 hours during which the excreta voided was discarded. After the expiration of the 24-hour starvation, each bird was dosed with 50ml of warm glucose solution to reduce stress. They were then deprived of feed for another 24 hours, making a total of 48 hours starvation period. Total excreta voided per bird during the last (24h) phase of feed starvation was collected, weighed and used for the estimation of endogenous losses. The excreta sample per bird was oven dried at 60°C until the weight was consistent; the samples were then pooled together by group and ground to pass through 0.1 mm sieve. Excreta samples (from fed and starved birds) were assayed for gross energy according to Sibbald (1980).

The following equations were used to calculate apparent metabolizable energy (AME), nitrogen corrected apparent metabolizable energy (AMEn), true metabolizable energy (TME), and nitrogen corrected true metabolizable energy (TMEn) of test samples.

$$\text{AM E/g of feed} = \frac{[(\text{Fi} \times \text{GEf}) - (\text{E} \times \text{GEe})]}{\text{Fi}}$$

where: Fi is the feed intake (g on dry matter basis), E is quantity of excreta output (g on dry matter basis), GEf is the gross energy (MJ/kg) of feed and GEe the gross energy (MJ/kg) of excreta.

$$\text{AM E/g of feed} = \frac{\{[(\text{Fi} \times \text{GEf}) - (\text{E} \times \text{GEe})] - (\text{NR} \times 36.5)\}}{\text{Fi}}$$

where: nitrogen retention (NR) = (Fi × Nf) − (E × Ne). Nf is the nitrogen content (g/kg) of feed, Ne is the nitrogen content (g/kg) of excreta.

$$\text{TM E/g of feed} = \frac{\{[(\text{Fi} \times \text{GEf}) - (\text{E} \times \text{GEe})] + (\text{FEm} + \text{UEe})\}}{\text{Fi}}$$

where: FEm is metabolic faecal energy (kJ) (calculated from gross energy of excreta from endogenous loss), and UEe is endogenous urinary energy (kJ) (This is assumed zero since urine and faeces are passed together).

$$\text{TME n/g of feed} = \frac{\{[(\text{Fi GEf}) - (\text{E} \times \text{GEe})] - (\text{NR} \times \text{K})\} + \{(\text{FEm} + \text{UEe}) + (\text{NRo} \times 36.5)\}}{\text{Fi}}$$

where: NR and NRo are estimates of nitrogen retention for fed and starved birds, respectively.

**Carcass Measurements**

At 8th week, six experimental birds per treatment (two per replicate) were selected and weighed for carcass measurements. Prior to slaughtering, the birds were starved overnight to empty their crops. They were then slaughtered by neck slitting, allowed to bleed, scalded in warm water and de-feathered. Dressed weights and weights of the cut parts and organs were thereafter taken. Weights of the cut parts and organs were expressed as percentage of live weights while the dressed percentage was calculated using the formula:

$$\text{Dressed Percentage}(\%) = \frac{\text{Dressed weight(g)}}{\text{Live weight(g)}} \times 100$$

**Statistical Analysis**

Data generated were analysed by the analysis of variance technique using the Statistical Analysis System computer package (Statistical Analysis System Institute [SAS Institute], 1999) to separate the main effects of using different varieties of cassava and different additives. The interaction effect between the white or yellow cassava varieties and the type of additive (no additive, amino acid supplementation, exogenous enzyme, or combination of amino acid and enzyme) was also determined. Differences between significant mean values were separated using Duncan's multiple range test (Duncan, 1955).

Statistical Model:

$$Y_{ijk} = \mu + A_i + B_j + (AB)_{ij} + E_{ijk}$$

where:

$Y_{ijk}$ = observed values of dependent variable

$\mu$ = population mean

$A_i$ = main effect of cassava (White and Yellow)

$B_j$ = main effect of additive (Non, Amino acid, Enzyme, Amino acid + Enzyme)

$(AB)_{ij}$ = interaction effect of cassava varieties and additives

$E_{ij}$ = random residual error

## RESULTS

The chemical composition of white cassava (WCRM) and yellow cassava root meal (YCRM) (average of four determinations)

as shown in Table 1 revealed that the dry matter content of the two cassava varieties used in this study differed, with values of 90.42% and 91.33% for WCRM and YCRM, respectively. White cassava root meal recorded higher values for crude fat and crude fibre content while 3.56% crude protein in YCRM was higher than 2.20% crude protein recorded for WCRM. The ash and β-carotene content (2.90% and 349.01 µg/100 g, respectively) obtained for YCRM were higher than the values obtained for ash and β-carotene content in WCRM. The NFE value recorded for WCRM (83.77%) was higher than 82.98% NFE recorded for YCRM. White cassava root meal showed higher values for NDF (26.59%), ADF (15.58%) and ADL (3.37%). It also had 2.66 mg/kg HCN content while the HCN content of YCRM was 2.54 mg/kg. Values of the minerals Ca, P, Cu, Mg, and Mn measured in YCRM were 3.55, 0.80, 0.28, 0.40, and 0.140, respectively, which were higher than the mineral contents recorded in WCRM except for the value of Zn which was the same as that obtained in YCRM. The gross energy value obtained for WCRM (3537.10 kcal/kg) was higher than the gross energy recorded for YCRM.

**Main Effect of Cassava Varieties and Additives on Nutrient Digestibility of Starter and Finisher Broilers**

The result of the main effects of cassava varieties and additives on nutrient digestibility of starter and finisher broilers as shown in Table 3 revealed that the two cassava varieties had no significant ($p>0.05$) effect on dry matter digestibility (DMD), crude protein retention (CPR), ether extract digestibility (EED), crude fibre digestibility (CFD), ash digestibility (ASHD), and nitrogen free extract digestibility (NFED) of the starter broilers. The various additives also had no significant ($p>0.05$) effect on dry matter digestibility (DMD), crude protein retention (CPR), ether extract digestibility (EED), crude fibre digestibility (CFD), ash digestibility (ASHD), and nitrogen free extract digestibility (NFED) of the broilers at the starter phase.

The result of main effects of cassava varieties and additives on nutrient digestibility of finisher broilers showed that birds fed white cassava variety had higher ($p<0.05$) DMD (73.89%), CPR (78.71%), EED (67.96%), CFD (64.25%), ASHD (69.89%) and NFED (67.04%) values. Main effect of additives showed that finisher broilers fed cassava diets + amino acids + enzyme had the least values ($p<0.05$) for DMD, EED, CFD, ASHD and NFED (71.96%, 64.74%, 59.66%, 63.85% and 65.25%). DMD, EED and CFD values obtained for finisher broilers fed cassava diets with no additive, those fed cassava diets with amino acid, and those fed cassava diets with enzyme were not significantly different ($p>0.05$).

**Interaction Effect of Cassava Varieties and Additives on Nutrient Digestibility of Starter and Finisher Broilers**

The result of the interaction effects of cassava varieties and additives on nutrient digestibility of starter and finisher broilers

Table 3
*Main effects of cassava varieties and additives on nutrient digestibility of starter and finisher broilers*

| Parameters (%) | Varieties | | | | Additives | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | White | Yellow | SEM | P-value | Control | A.A | Enzyme | A.A + Enz | SEM | P-value |
| *Starter Phase* | | | | | | | | | | |
| Dry matter digestibility | 74.34 | 74.97 | 0.30 | 0.413 | 74.51 | 74.93 | 74.35 | 74.83 | 0.41 | 0.813 |
| Crude protein digestibility | 76.36 | 76.76 | 0.20 | 0.357 | 76.49 | 76.73 | 76.15 | 76.88 | 0.25 | 0.638 |
| Ether extract digestibility | 72.77 | 72.49 | 0.38 | 0.572 | 72.71 | 72.57 | 71.94 | 73.29 | 0.52 | 0.678 |
| Crude fibre digestibility | 58.22 | 61.01 | 1.35 | 0.218 | 58.59 | 60.17 | 60.50 | 59.19 | 1.92 | 0.543 |
| Ash digestibility | 71.38 | 73.23 | 1.11 | 0.511 | 70.59 | 73.69 | 72.67 | 72.26 | 1.46 | 0.711 |
| Nitrogen free extract digestibility | 66.23 | 66.71 | 0.19 | 0.543 | 66.28 | 66.68 | 66.36 | 66.56 | 0.26 | 0.588 |
| Average feed intake/bird (g) | 1367.50 | 1358.52 | 31.30 | 0.812 | 1278.79$^{b}$ | 1338.18$^{b}$ | 1460.07$^{a}$ | 1375.00$^{ab}$ | 35.99 | 0.020 |
| *Finisher Phase* | | | | | | | | | | |
| Dry matter digestibility | 73.89$^{a}$ | 72.63$^{b}$ | 0.29 | 0.026 | 73.04$^{b}$ | 74.03$^{a}$ | 74.03$^{a}$ | 71.96$^{c}$ | 0.34 | 0.000 |
| Crude protein digestibility | 78.71$^{a}$ | 77.51$^{b}$ | 0.13 | 0.021 | 78.31$^{a}$ | 78.02$^{b}$ | 78.16$^{ab}$ | 77.94$^{b}$ | 0.27 | 0.024 |
| Ether extract digestibility | 67.96$^{a}$ | 65.86$^{b}$ | 0.46 | 0.031 | 67.28$^{a}$ | 67.66$^{a}$ | 67.96$^{a}$ | 64.74$^{b}$ | 0.59 | 0.001 |
| Crude fibre digestibility | 64.25$^{a}$ | 61.86$^{b}$ | 0.82 | 0.012 | 63.04$^{a}$ | 64.98$^{a}$ | 64.54$^{a}$ | 59.66$^{b}$ | 0.94 | 0.017 |
| Ash digestibility | 69.89$^{a}$ | 65.75$^{b}$ | 0.95 | 0.035 | 66.58$^{b}$ | 70.94$^{a}$ | 69.92$^{a}$ | 63.85$^{c}$ | 1.03 | 0.000 |
| Nitrogen free extract digestibility | 67.04$^{a}$ | 65.58$^{b}$ | 0.28 | 0.034 | 65.97$^{b}$ | 66.44$^{b}$ | 67.59$^{a}$ | 65.25$^{c}$ | 0.35 | 0.000 |
| Average feed Intake/bird (g) | 3180.06 | 3124.23 | 33.87 | 0.250 | 3217.62 | 3081.58 | 3167.33 | 3142.05 | 46.75 | 0.263 |

*Note.* All values within rows having the same or no superscripts are not significantly different (p>0.05)
A.A = Amino acids (Methionine and Lysine), Enzyme = Cellulase, A.A + Enz = (Methionine and Lysine + Cellulase)

is shown in Table 4. Starter broilers fed yellow cassava diet with amino acids + enzyme, recorded higher ($p<0.05$) values for DMD, CPR, EED, CFD, ASHD, and NFED (76.31%, 77.82%, 74.40%, 65.85%, 77.42% and 67.52% in that order) while starter broilers fed white cassava diet with amino acids + enzyme had lower values except for EED.

The result of the interaction effects of cassava varieties and additives on nutrient digestibility of finisher broilers revealed that finisher broilers fed yellow cassava diet with amino acids + enzyme had lower values ($p<0.05$) of DMD, CPR, EED, ASHD, CFD, and NFED. Finisher broilers fed white cassava supplemented with amino acids had higher values ($p<0.05$) of DMD (74.73%), EED (69.09%) and ASHD (73.14%) while finisher broilers fed white cassava with no additive had higher ($p<0.05$) CPR (78.88%) value. NFED (68.77%) value was highest ($p<0.05$) for birds fed white cassava diets with enzyme.

**Main Effect of Cassava Varieties and Additives on Metabolizable Energy of Starter and Finisher Broilers**

The result of main effects of cassava varieties and additives on the metabolizable energy of starter and finisher broilers is presented in Table 5. The result showed that birds that were fed with white cassava root meal at the starter phase had higher ($p<0.05$) AME, AMEn, TME, and TMEn (13.11 MJ/kg, 11.67 MJ/kg, 13.22 MJ/kg and 11.78 MJ/kg respectively) values than the birds that were fed the yellow variety. The Starter broilers fed cassava diets with enzyme recorded highest ($p<0.05$) values for AME (13.69 MJ/kg), AMEn (12.24 MJ/kg), TME (13.80 MJ/kg), and TMEn (12.35 MJ/kg) while the other birds fed cassava diets with other additives had lower values of AME, AMEn, TME, and TMEn.

The result of main effects of cassava varieties and additives on the metabolizable energy of finisher broilers showed that no significant ($p>0.05$) effect was observed with the use of white and yellow cassava root meals on AME, AMEn, TME, and TMEn values for the birds. Finisher broilers fed cassava diets with amino acids recorded the highest ($p<0.05$) values for AME (13.64 MJ/kg), AMEn (12.57 MJ/kg), TME (13.75 MJ/kg), and TMEn (12.68 MJ/kg) while birds fed cassava diets with amino acids + enzyme had the least AME, AMEn, TME, and TMEn values.

**Interaction Effect of Cassava Varieties and Additives on Metabolizable Energy of Starter and Finisher Broilers**

The result of the interaction effects of cassava varieties and additives on the metabolizable energy of starter and finisher broilers is shown in Table 6. At the starter phase, birds fed white cassava with enzyme recorded highest ($p<0.05$) values for AME, AMEn, TME, and TMEn (14.72 MJ/kg, 13.28 MJ/kg, 14.83 MJ/kg and 13.38 MJ/kg respectively), while lowest values of AME, AMEn, TME, and TMEn were recorded with birds fed yellow cassava with no additive.

Table 4
*Interaction effects of cassava varieties and additives on nutrient digestibility of starter and finisher broilers*

| Parameters (%) | White cassava | | | | Yellow cassava | | | | SEM | P-value |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | A.A | Enzyme | A.A + Enz | Control | A.A | Enzyme | A.A + Enz | | |
| *Starter Phase* | | | | | | | | | | |
| Dry matter digestibility | 74.46$^{bc}$ | 75.26$^{ab}$ | 74.27$^{bc}$ | 73.34$^{c}$ | 74.56$^{bc}$ | 74.59$^{bc}$ | 74.43$^{bc}$ | 76.31$^{a}$ | 0.22 | 0.043 |
| Crude protein digestibility | 76.47$^{bc}$ | 76.95$^{ab}$ | 76.10$^{bc}$ | 75.93$^{c}$ | 76.50$^{bc}$ | 76.50$^{bc}$ | 76.20$^{bc}$ | 77.82$^{a}$ | 0.15 | 0.044 |
| Ether extract digestibility | 73.19$^{ab}$ | 73.22$^{ab}$ | 72.49$^{ab}$ | 72.17$^{ab}$ | 72.23$^{ab}$ | 71.92$^{b}$ | 71.39$^{b}$ | 74.40$^{a}$ | 0.27 | 0.019 |
| Crude fibre digestibility | 59.92$^{ab}$ | 60.18$^{ab}$ | 60.25$^{ab}$ | 52.53$^{c}$ | 57.26$^{bc}$ | 60.16$^{ab}$ | 60.76$^{ab}$ | 65.85$^{a}$ | 0.98 | 0.038 |
| Ash digestibility | 70.12$^{cd}$ | 75.84$^{ab}$ | 72.44$^{abcd}$ | 67.10$^{d}$ | 71.06$^{bcd}$ | 71.53$^{bcd}$ | 72.90$^{abc}$ | 77.42$^{a}$ | 0.79 | 0.048 |
| Nitrogen free extract digestibility | 66.19$^{bc}$ | 66.83$^{ab}$ | 66.31$^{bc}$ | 65.60$^{c}$ | 66.36$^{bc}$ | 66.52$^{bc}$ | 66.41$^{bc}$ | 67.52$^{a}$ | 0.14 | 0.043 |
| Average feed Intake/bird (g) | 1302.73$^{bc}$ | 1405.15$^{ab}$ | 1463.47$^{a}$ | 1298.64$^{bc}$ | 1254.85$^{c}$ | 1271.21$^{bc}$ | 1456.67$^{a}$ | 1451.36$^{a}$ | 21.80 | 0.009 |
| *Finisher Phase* | | | | | | | | | | |
| Dry matter digestibility | 73.70$^{bc}$ | 74.73$^{a}$ | 74.33$^{ab}$ | 72.82$^{cd}$ | 73.38$^{d}$ | 73.34$^{c}$ | 73.74$^{bc}$ | 71.09$^{e}$ | 0.24 | 0.032 |
| Crude protein digestibility | 78.88$^{a}$ | 78.76$^{ab}$ | 78.62$^{b}$ | 78.57$^{b}$ | 77.75$^{c}$ | 77.28$^{d}$ | 77.70$^{c}$ | 77.31$^{d}$ | 0.13 | 0.001 |
| Ether extract digestibility | 67.89$^{abc}$ | 69.09$^{a}$ | 68.46$^{ab}$ | 66.38$^{c}$ | 66.66$^{bc}$ | 66.22$^{c}$ | 67.45$^{abc}$ | 63.09$^{d}$ | 0.40 | 0.041 |
| Crude fibre digestibility | 63.85$^{a}$ | 65.45$^{a}$ | 65.83$^{a}$ | 61.85$^{a}$ | 62.22$^{a}$ | 64.51$^{a}$ | 63.26$^{a}$ | 57.46$^{b}$ | 0.63 | 0.000 |
| Ash digestibility | 69.82$^{b}$ | 73.14$^{a}$ | 70.77$^{ab}$ | 65.87$^{c}$ | 63.33$^{d}$ | 68.75$^{b}$ | 69.07$^{b}$ | 61.83$^{d}$ | 0.79 | 0.015 |
| Nitrogen free extract digestibility | 66.48$^{bc}$ | 66.88$^{b}$ | 68.77$^{a}$ | 66.04$^{c}$ | 65.47$^{d}$ | 65.99$^{cd}$ | 66.41$^{bc}$ | 64.47$^{e}$ | 0.25 | 0.003 |
| Average feed Intake/bird (g) | 3195.64 | 3143.33 | 3232.33 | 3148.94 | 3239.61 | 3019.82 | 3102.33 | 3135.15 | 24.46 | 0.395 |

$^{a,b,c,d}$means with the same superscripts along the rows are not significantly different ($p<0.05$)
A.A = Amino acids (Methionine and Lysine), Enzyme = Cellulase, A.A + Enz = (Methionine and Lysine + Cellulase)

Table 5
*Main effects of cassava varieties and additives on the metabolizable energy of starter and finisher broilers*

| Parameters (MJ/Kg) | Varieties | | | | Additives | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | White | Yellow | SEM | P-value | Control | A.A | Enzyme | A.A + Enz | SEM | P-value |
| *Starter Phase* | | | | | | | | | | |
| AME | 13.11[a] | 12.56[b] | 0.16 | 0.033 | 12.52[b] | 12.55[b] | 13.69[a] | 12.58[b] | 0.14 | 0.000 |
| $AME_n$ | 11.67[a] | 11.11[b] | 0.16 | 0.025 | 11.07[b] | 13.11[b] | 12.24[a] | 11.13[b] | 0.14 | 0.000 |
| TME | 13.22[a] | 12.67[b] | 0.16 | 0.019 | 12.63[b] | 12.67[b] | 13.80[a] | 12.69[b] | 0.14 | 0.000 |
| $TME_n$ | 11.78[a] | 11.22[b] | 0.16 | 0.021 | 11.18[b] | 11.22[b] | 12.35[a] | 11.25[b] | 0.14 | 0.000 |
| *Finisher Phase* | | | | | | | | | | |
| AME | 13.56 | 13.55 | 0.02 | 0.185 | 13.55[b] | 13.64[a] | 13.56[b] | 13.48[c] | 0.01 | 0.000 |
| AMEn | 12.46 | 12.47 | 0.02 | 0.319 | 12.46[b] | 12.57[a] | 12.45[b] | 12.38[c] | 0.02 | 0.000 |
| TME | 13.67 | 13.66 | 0.02 | 0.526 | 13.66[b] | 13.75[a] | 13.67[b] | 13.59[c] | 0.01 | 0.000 |
| TMEn | 12.57 | 12.58 | 0.02 | 0.811 | 12.57[b] | 12.68[a] | 12.57[b] | 12.49[c] | 0.02 | 0.000 |

[a,b] means with the same superscripts along the rows are not significantly different ($p<0.05$)
AME = Apparent Metabolizable Energy; $AME_n$ = Apparent Metabolizable Energy corrected for Nitrogen Retention; TME = True Metabolizable Energy; $TME_n$= True Metabolizable Energy corrected for Nitrogen Retention

Table 6
*Interaction effects of cassava varieties and additives on the metabolizable energy of starter and finisher broilers*

| Parameters (MJ/Kg) | WCRM | | | | YCRM | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | A.A | Enzyme | A.A + Enz | Control | A.A | Enzyme | A.A + Enz | SEM | P-value |
| Starter Phase | | | | | | | | | | |
| AME | 12.67[b] | 12.49[e] | 14.72[a] | 12.57[d] | 12.36[f] | 12.62[c] | 12.66[bc] | 12.58[d] | 0.15 | 0.024 |
| $AME_n$ | 11.22[b] | 11.05[e] | 13.28[a] | 11.12[d] | 10.92[f] | 11.17[c] | 11.21[b] | 11.14[cd] | 0.15 | 0.026 |
| TME | 12.78[b] | 12.60[e] | 14.83[a] | 12.68[d] | 12.47[f] | 12.74[c] | 12.78[b] | 12.70[d] | 0.15 | 0.013 |
| $TME_n$ | 11.33[b] | 11.16[e] | 13.38[a] | 11.23[d] | 11.03[f] | 11.28[c] | 11.32[b] | 11.26[cd] | 0.15 | 0.018 |
| Finisher Phase | | | | | | | | | | |
| AME | 13.54[c] | 13.69[a] | 13.54[c] | 13.47[e] | 13.55[c] | 13.59[b] | 13.58[b] | 13.49[d] | 0.01 | 0.001 |
| AMEn | 12.44[d] | 12.62[a] | 12.43[de] | 12.35[f] | 12.48[c] | 12.51[b] | 12.48[b] | 12.41[e] | 0.02 | 0.001 |
| TME | 13.65[d] | 13.80[a] | 13.66[cd] | 13.57[f] | 13.66[cd] | 13.70[b] | 13.69[bc] | 13.60[e] | 0.01 | 0.003 |
| TMEn | 12.56[d] | 12.73[a] | 12.55[d] | 12.46[f] | 12.59[c] | 12.62[b] | 12.59[e] | 12.51[e] | 0.02 | 0.000 |

[a,b,c,d,e,f] means with the same superscripts along the rows are not significantly different ($p<0.05$)
AME = Apparent Metabolizable Energy; $AME_n$ = Apparent Metabolizable Energy corrected for Nitrogen Retention; TME = True Metabolizable Energy; $TME_n$= True Metabolizable Energy corrected for Nitrogen Retention

Finisher broilers fed white cassava with amino acid recorded the highest ($p<0.05$) values for AME, AMEn, TME, and TMEn while finisher broilers fed white cassava with amino acids + enzyme had the least AME, AMEn, TME, and TMEn values (13.49 MJ/kg, 12.41 MJ/kg, 13.60 MJ/kg, and 12.51 MJ/kg respectively).

### Main Effect of Cassava Varieties and Additives on Carcass Yield and Cut Parts of Broilers

Table 7 shows the result of main effects of cassava varieties and additives on the carcass yield and cut parts of broilers. It was observed that white and yellow varieties of cassava had no significant ($p>0.05$) effect on live weight, plucked weight, eviscerated weight, dressed weight, dressed percentage, breast, back, drumstick, thigh, neck, wings, head and shanks of the birds across the treatments. The main effect of additive was also not significant ($p>0.05$) on live weight, plucked weight, eviscerated weight, dressed weight, dressed percentage, breast, back, drumstick, thigh, neck, wings, head, and shanks of the experimental birds.

### Interaction Effect of Cassava Varieties and Additives on Carcass Yield and Cut Parts of Broilers

The result of the interaction effects of cassava varieties and additives on the carcass yield and cut parts of broilers (8 weeks) are as presented in Table 8. The interaction between cassava variety and inclusion of additives were not significant ($p>0.05$) for plucked weight, eviscerated weight, dressed weight, dressed percentage, breast, back, drumstick, thigh, neck, wings, and shanks of the birds. Broilers fed white cassava diet with amino acids had higher ($p<0.05$) live weight value (1946.33g) while the broilers fed white cassava with no additive recorded lower live weight value (1878.33g).

## DISCUSSION

### Main Effect of Cassava Varieties and Additives on Nutrient Digestibility and Metabolizable Energy of Starter and Finisher Broilers

Yellow cassava varieties investigated in this study showed poor nutrient digestibilities when compared to the white variety. This implies that it did not contribute any digestible benefit to the birds. The findings on the main effect of additives showed that dietary supplementation with either amino acids or enzyme significantly improved nutrient digestibility at the finisher phase. Amino acid supplementation in turkeys has been reported by Oso et al. (2017) to improve nutrient digestibility. Amino acids added make-up for the shortages of Sulphur amino acids depleted during thiocyanate detoxification. Enzyme supplementation has been reported to break down fibrous component in cassava thereby improving its digestibility (Belewu & Banjo, 1999).

White cassava variety recorded higher metabolizable energy values when compared with the yellow variety at the starter phase. The fact that there was no difference in the metabolizable energy values of finishing broilers fed either white or yellow cassava implied that both yielded similar energy values.

Table 7
*Main effects of cassava varieties and additives on the carcass yield and cut parts of broilers (8 weeks)*

| Parameters | Varieties | | | | Additives | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | White | Yellow | SEM | P-value | Control | A.A | Enzyme | A.A + Enz | SEM | P-value |
| Live weight (g) | 1923.84 | 1906.42 | 36.16 | 0.170 | 1889.50 | 1921.33 | 1930.17 | 1919.50 | 50.30 | 0.061 |
| Plucked weight (g) | 1798.65 | 1798.00 | 39.46 | 0.251 | 1780.24 | 1800.38 | 1809.21 | 1803.49 | 55.70 | 0.058 |
| Eviscerated weight (g) | 1554.42 | 1562.21 | 33.86 | 0.091 | 1544.26 | 1567.24 | 1555.99 | 1565.77 | 50.60 | 0.112 |
| Dressed weight (g) | 1190.78 | 1217.91 | 27.27 | 0.359 | 1197.60 | 1199.23 | 1217.25 | 1203.3 | 41.30 | 0.073 |
| Dressed % | 64.99 | 67.25 | 1.02 | 0.600 | 66.51 | 65.78 | 66.39 | 65.81 | 1.56 | 0.253 |
| *Cut Parts* | | | | | | | | | | |
| *(% live weight)* | | | | | | | | | | |
| Breast | 17.33 | 18.19 | 0.48 | 0.580 | 18.23 | 17.64 | 17.38 | 17.79 | 0.69 | 0.395 |
| Back | 13.90 | 14.11 | 0.47 | 0.701 | 14.24 | 13.62 | 14.38 | 13.77 | 0.69 | 0.721 |
| Drumstick | 9.50 | 10.00 | 0.22 | 0.630 | 9.47 | 10.09 | 9.76 | 9.66 | 0.33 | 0.819 |
| Thigh | 10.02 | 10.20 | 0.27 | 0.419 | 10.10 | 9.81 | 10.38 | 10.14 | 0.35 | 0.703 |
| Neck | 3.81 | 3.59 | 0.10 | 0.725 | 3.61 | 3.88 | 3.52 | 3.79 | 0.14 | 0.634 |
| Wings | 7.50 | 7.83 | 0.14 | 0.182 | 7.74 | 7.72 | 7.65 | 7.55 | 0.21 | 0.659 |
| Head | 2.50 | 2.52 | 0.08 | 0.360 | 2.41 | 2.57 | 2.66 | 2.38 | 0.10 | 0.710 |
| Shank | 4.21 | 4.36 | 0.17 | 0.714 | 3.99 | 4.52 | 4.62 | 4.01 | 0.21 | 0.439 |

Table 8
*Interaction effects of cassava varieties and additives on the carcass yield and cut parts of broilers (8 weeks)*

| Parameters | WCRM | | | | YCRM | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | A.A | Enzyme | A.A + Enz | Control | A.A | Enzyme | A.A + Enz | SEM | P-value |
| Live weight (g) | 1900.67$^{c}$ | 1946.33$^{a}$ | 1936.67$^{ab}$ | 1911.67$^{abc}$ | 1878.33$^{abc}$ | 1896.33$^{bc}$ | 1923.67$^{bc}$ | 1927.33$^{ab}$ | 26.10 | 0.000 |
| Plucked weight (g) | 1785.90 | 1801.08 | 1818.43 | 1789.19 | 1774.57 | 1799.68 | 1799.18 | 1817.78 | 27.50 | 0.072 |
| Eviscerated weight(g) | 1548.90 | 1588.04 | 1535.00 | 1545.78 | 1539.66 | 1546.43 | 1576.98 | 1585.76 | 24.80 | 0.601 |
| Dressed weight(g) | 1178.60 | 1166.59 | 1187.37 | 1230.54 | 1216.59 | 1231.86 | 1247.12 | 1176.06 | 20.00 | 0.781 |
| Dressed % | 64.94 | 63.35 | 64.54 | 67.65 | 68.08 | 68.22 | 68.24 | 64.45 | 0.74 | 0.912 |
| *Cut Parts* | | | | | | | | | | |
| *(% live weight)* | | | | | | | | | | |
| Breast | 17.48 | 17.03 | 16.47 | 18.36 | 18.98 | 18.26 | 18.28 | 17.21 | 0.34 | 0.548 |
| Back | 14.18 | 12.62 | 14.22 | 14.57 | 14.30 | 14.61 | 14.54 | 12.96 | 0.33 | 0.587 |
| Drumstick | 9.03 | 9.95 | 9.63 | 9.37 | 9.91 | 10.23 | 9.90 | 9.96 | 0.17 | 0.631 |
| Thigh | 9.96 | 9.73 | 9.84 | 10.55 | 10.24 | 9.89 | 10.93 | 9.72 | 0.19 | 0.712 |
| Neck | 3.81 | 3.89 | 3.52 | 4.03 | 3.41 | 3.87 | 3.51 | 3.55 | 0.07 | 0.635 |
| Wings | 7.55 | 7.34 | 7.62 | 7.49 | 7.93 | 8.10 | 7.67 | 7.62 | 0.10 | 0.583 |
| Head | 2.29$^{b}$ | 2.61$^{ab}$ | 2.83$^{a}$ | 2.27$^{b}$ | 2.53$^{ab}$ | 2.53$^{ab}$ | 2.50$^{ab}$ | 2.50$^{ab}$ | 0.05 | 0.023 |
| Shank | 4.06 | 4.30 | 4.63 | 3.85 | 3.91 | 4.73 | 4.62 | 4.18 | 0.12 | 0.659 |

$^{a,b,c}$ means with the same superscripts along the rows are not significantly different ($p<0.05$)

**Interaction Effect of Cassava Varieties and Additives on Nutrient Digestibility and Metabolizable Energy of Starter and Finisher Broilers**

Starting broilers fed diet containing yellow cassava root meal (YCRM) supplemented with A.A + Enz recorded improved nutrient digestibility. Obviously, the amino acid supplementation and enzyme inclusion would have improved utilization of protein at this stage. The improvement could also be attributed to cassava variety used. Yellow cassava root meal has lower fibre content that could lead to improved digestibility and nutrient utilization compared to white variety of cassava (Isikwenu et al., 2000). In comparison with white cassava variety, yellow cassava contains higher quantity of β-carotene which may be indicative of its higher potential antioxidant roles, enhanced immune system (Navara & Hills, 2003) and reduced risk of degenerative diseases (Cooper et al., 1999). All these can also contribute to improved nutrient digestibility of the yellow cassava variety. At the finishing phase, the broilers fed white cassava root meal (WCRM) supplemented with amino acids recorded better digestibility of dry matter, fat, ash and crude fibre. Supplementing the diet with amino acids could have improved the normal functioning of the digestive system of the birds thereby enhancing improved nutrient utilization. The superiority of YCRM at the starting phase was not sustained at the finishing phase because the younger birds at the starting phase required better mix of nutrient reported to be present in YCRM, whereas at the finishing phase, the need for this nutrient would have reduced. Coupled with the stronger digestive system of the birds at the finishing phase, addition of amino acids to white cassava would have improved the digestibility and absorption of white cassava at this phase. Oso et al. (2017) hinted that amino acids supplementation improved nutrient digestibility and absorption in grower and finisher turkeys.

Improved metabolizable energy values of starter broilers fed WCRM diet supplemented with enzyme when compared with other treatments could be due to higher energy [Gross energy (GE)] content of white cassava variety and exogenous enzyme added. White cassava root meal in this study has been assayed to have more energy (GE) than YCRM. With appropriate enzyme supplementation of WCRM, more energy is released when compared to YCRM. Cellulase enzymes used in this study has been reported to degrade the fibre component of cassava product, thereby making more energy available to monogastric (Belewu & Banjo, 1999; Raji & Okeniyi, 1998). Previous studies also confirmed significant improvements in energy metabolizability of feed ingredient following enzyme supplementation (Adeola & Bedford, 2004; Meng & Slominski, 2005; Slominski, 2011).

Supplementation of WCRM with amino acids only at the finisher phase, resulted in improved metabolizable energy values as opposed to starter broilers which

needed enzyme supplementation of WCRM to elicit best energy metabolizability. As the birds grew older, their gastro-intestinal tract (GIT) became more mature to handle fibrous components of the feed which the starting birds could not handle because of their immature digestive system. Improved metabolizable energy values of WCRM following limiting amino acid supplementation at the finishing phase could also be due to the high energy (GE) content of WCRM and amino acid supplementation which made up for the shortage of protein content and poor amino acid profile in cassava. The mechanism through which limiting amino acid supplementation improves energy metabolizability could be attributed to some biochemical reactions which occurred during cassava processing. Cyanide contained in cassava root is converted to a less toxic thiocyanate during processing using the enzyme rhodnase. This biochemical conversion to thiocyanate utilizes the constituent sulphur containing amino acid as sulfur donor (Cardoso et al., 2005) thus making sulphur amino acid to be grossly deficient in cassava-based diets. Hence, supplemental amino acid used in this study greatly made allowance for the shortage that would have been created, thereby harnessing the rich energy potential of WCRM maximally. Improved energy metabolizability of cassava root meal for meat-type cockerels had also been reported by Oso et al. (2015) following amino acid supplementation and solid-state fermentation.

**Main and Interaction Effects of Cassava Varieties and Additives on The Carcass Yield and Cut Parts of Broilers**

According to Adeyemi et al. (2008) and Agunbiade et al. (2002), the breast meat, drumstick and thigh are the most expensive commercial cuts of the chicken, which give a picture of the carcass meatiness and eventually revenue yield. Cassava varieties and additives used in this study showed no influence on plucked weight, eviscerated weight, dressed weight, dressed percentage, breast, back, drumstick, thigh and wings of the birds. This is at variance with the report that dressed weight, thigh and drumstick weight were influenced by dietary treatments (Adeyemi et al., 2012) but agrees with the observations of Eruvbetine et al. (2003) and George and Sese (2012) that dietary treatments had no influence on carcass quality characteristics. Rahmatnejad et al. (2011) reported that addition of commercial multi-enzyme did not improve dressed percentage, yield of breast, thigh and wing components.

The insignificant effect of diets on heart, kidney, spleen, bile, whole gizzard, empty gizzard, and whole intestine values of the broilers across the dietary treatments indicated that there were no abnormalities or pathological lesions in these organs. This aligns with the report of Omojola and Adesehinwa (2007) that the inclusion of exogenous enzyme in broilers fed cassava meal diets showed no effect on the relative weights of kidney, gizzard and heart. The use of protein concentrates with a good balance of amino acids in broiler diets containing

cassava meal showed no significant effect on the weights of the internal organs like liver, kidney, heart and spleen (Adeyemi et al., 2012).

## CONCLUSION

It was concluded from this study that WCRM favoured improved nutrient utilization and digestibility than YCRM. Further supplementation of YCRM with both amino acids + enzyme at starter phase and WCRM with amino acids at finisher phase improved nutrient digestibility of broilers. Amino acid supplementation of cassava diets (WCRM, YCRM) augmented the constituent poor amino acid profile in cassava root and hence improved the nutrient utilization and digestibility. Meanwhile, for optimal utilization of YCRM, both amino acids and enzyme need to be supplemented. Enzyme and amino acid supplementation of WCRM at the starter and finisher phases respectively of broilers resulted in improved metabolizable energy values than in YCRM. Supplementing diet containing WCRM with enzyme assisted in the breakdown of the constituent fibre, increased available energy, thus yielding improved energy metabolizability. Supplementing diet containing WCRM with amino acid made allowance for the shortage of protein content and poor amino acid profile in cassava and harnessed the rich energy potential of WCRM maximally. This resulted in improved metabolizable energy values.

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# The Influences of Water Quality on Fish Occurrences in Kuala Mai, Pahang River and Ulu Tembeling, Tembeling River, Pahang, Malaysia

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## ABSTRACT

This study assessed the fish community structures and influences of water quality on fish occurrences in Pahang and Tembeling rivers, Pahang. Fish samplings and water quality measurements were conducted in Kuala Mai, Pahang River and Ulu Tembeling, Tembeling River from 12 and eight different sampling points, from October 2007 to September 2008, and August 2006 to August 2007, respectively. The fish diversity, richness and evenness indices were determined, while the water quality parameters were compared for both rivers. Multivariate analyses were then used to explore the effects of water physicochemical parameters on the fish occurrences. A total of 2,391 individuals were collected from this study, comprising of 20 families and 65 species of fish. Using the gill nets, cast nets and fishing rods, a total of 55 fish species from 17 families were recorded in Kuala Mai, Pahang River, compared to 47 species from 15 families in Ulu Tembeling, Tembeling River. There were significant differences ($p < 0.05$) for fish diversity (*H* and *1-D*), but not for fish evenness (*e*) and richness (*D*) between both rivers. The mean water temperature, ammonia nitrogen and total suspended solids were significantly different ($p < 0.05$) in both rivers. Apart from the influences of pH, alkalinity and phosphate in both rivers, the results showed that the temperature, dissolved oxygen and conductivity were the major influencers on the fish occurrences in

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Kuala Mai, Pahang River, while ammonia nitrogen and total suspended solids in Ulu Tembeling, Tembeling River. Fish conservation and stock management efforts are urgently needed due to the decreasing number of near-threatened, endangered and critically endangered fish species in both rivers.

*Keywords*: Community structure, fish ecology, Pahang River, physicochemical parameters, Tembeling River

## INTRODUCTION

Fishes are highly beneficial in order to understand the condition of aquatic ecosystems and this could involve relating the fish abundance with water quality (Tsai et al., 2016). The importance of physicochemical parameters in freshwater ecosystems cannot be overemphasized since they not only influence, to a large extent, fish survival but also dictate their richness and distribution (Nyanti et al., 2018). An insight into the relationship between fish and its habitat is quintessential for effective management and conservation, because fish are subservient to the quality and dynamics of the water quality in which they reside (Paller et al., 2016).

With a length of 459 km, the Pahang River remains the longest in Peninsular Malaysia, meandering through a number of districts and townships. The Pahang River is divided into the Jelai and Tembeling rivers, which meet near Kuala Tembeling at Jerantut District. Pahang and Tembeling rivers are very important freshwater fish habitats in Peninsular Malaysia, as they provide useful natural capital for beneficial ecosystem services (Bhuiyan et al., 2014). In addition to their roles in the accommodation and breeding of aquatic life, the environment of these rivers also promotes aquaculture activities, especially of several commercially valuable freshwater fish species like *Pangasianodon* sp. and *Oreochromis* sp. (Zulkafli et al., 2015b). Locals specifically depend on these rivers as sources of protein, particularly from shrimp and fish, and in connection to this, as the main source of side income (Idris et al., 2016). More so, they also shelter some fish species which are already classified as endangered like *Fluvitrygon signifier* and *Probarbus jullieni* (Zulkafli et al., 2015b).

Tembeling River has a length of 153 km with a catchment area of 5,050 km², which is mainly covered by forests, rubber plantations, oil palm plantations, lakes, rivers, and marshes at 66%, 13%, 12%, and 9%, respectively (Zulkafli et al., 2015b). It is located in the upper Tembeling region and impacts essentially on the socio-economics of the people in the area, as being a source of food and transportation (Idris et al., 2016; Samah et al., 2013). Pahang and Tembeling rivers are also famous for tourism activities. For example, Tembeling River specifically flows along the National Park at Kuala Tahan, Pahang, which is beneficial to some of the locals in generating great income via eco-tourism activities including water-rafting and boat-riding (Shuib, 1995).

Several studies have enumerated the fish abundance (Hashim et al., 2012; Ismail et al., 2013; Nasruddin et al., 2014; Jalal et al., 2018; Sule et al., 2016; Zaid et al., 2018; Zulkafli et al., 2015a, 2015b, 2016a, 2016b), while others have

demonstrated the impacts of water quality on fish assemblages (Ikhwanuddin et al., 2016; Sule et al., 2018; Yusof et al., 2018; Zulkafli et al., 2018) of aquatic ecosystems around Malaysia. However, there is still a dearth of information on the dynamics of fish occurrences as dictated by water quality parameters in Pahang and Tembeling rivers. Thus, the objectives of this study are to determine the fish community structures and evaluate the influences of water physicochemical parameters on the fish occurrences, specifically in Kuala Mai, Pahang River and Ulu Tembeling, Tembeling River, Pahang, Malaysia.

## MATERIALS AND METHODS

### Study Area

The study was conducted at Kuala Mai, Pahang River in Temerloh District and Ulu Tembeling, Tembeling River in Jerantut District, Pahang, Malaysia (Figure 1). The samplings took place at monthly intervals from October 2007 to September 2008 (10 months) in Kuala Mai, Pahang River at 12 different sampling points, and from August 2006 to August 2007 (12 months) in Ulu Tembeling, Tembeling River at eight different sampling points (Table 1). All sites were accessible by road and located in the relatively undisturbed area where industrial

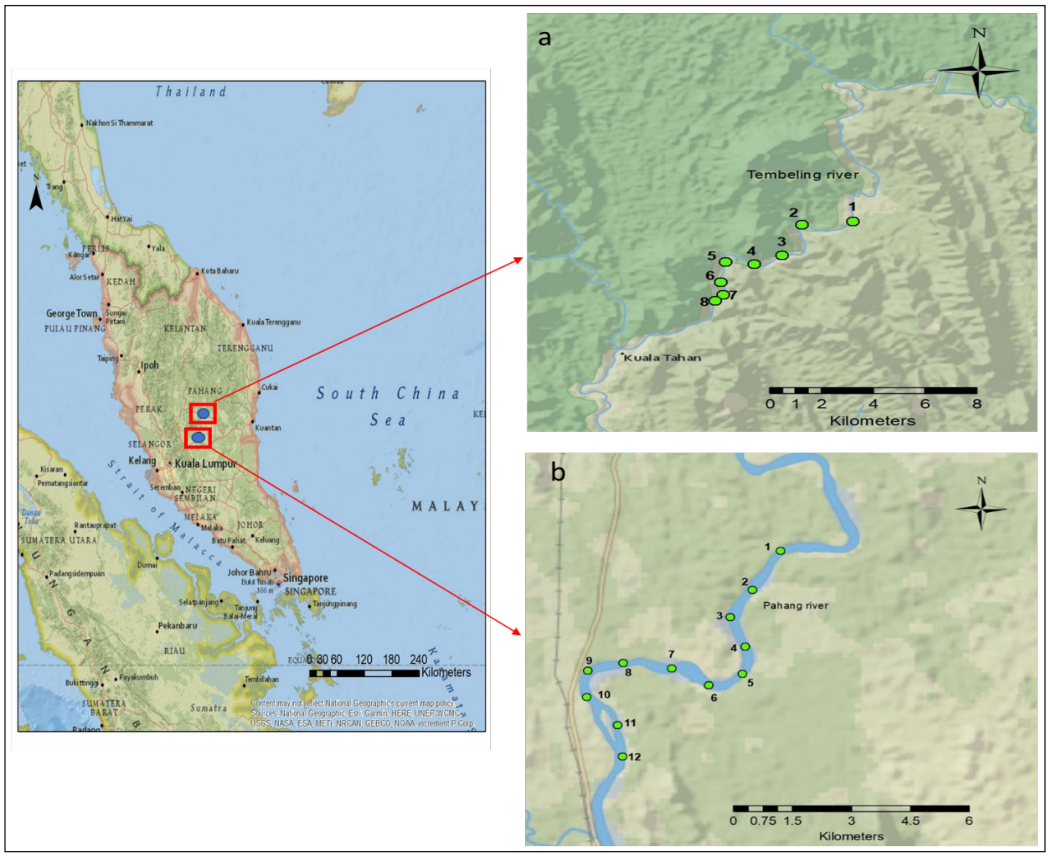


*Figure 1*. Sampling points location in (a) Ulu Tembeling, Tembeling River and (b) Kuala Mai, Pahang River

and agricultural activities were not visible. All of the sampling sites were characterized by a large river with deep slow reaches, a river bed that is usually covered with sand or gravel substrates, and no tidal influence.

Annual rainfall of the Pahang River is influenced by the northeast monsoon season, ranging from 1,609 mm to 2,132 mm, which occurs mostly between November and March of every year. This river empties into the South China Sea on the east coast of Peninsular Malaysia (Roseli et al., 2014). Situated near the Pahang National Park and with aboriginals occupying its banks at Ulu Tembeling, the Tembeling River is one of the main tributaries of Pahang River (Zulkafli et al., 2015b).

## Fish Sampling

In all sampling sites, three different sizes of drift and static gill nets (15 - 30 m long, 2.5 - 4.0 m wide) with mesh sizes ranging from 2.5 to 23.0 cm between knots were used. The drift and static gill nets were deployed between 7:00 - 10:00 a.m.. The static gill nets were maintained in position, checked at every 7 - 10 h, and hauled in after 24 h (Zulkafli et al., 2018). Traditional fishing gears, such as cast net and fishing rods (one and two for each gear), were also used in sites where the utilization of gill net was not suitable, and in order to diversify sampling techniques and types of collected fishes.

Table 1
*Coordinates of sampling points in Kuala Mai, Pahang River and Ulu Tembeling, Tembeling River*

| River | Point | Latitude | Longitude |
|---|---|---|---|
| Kuala Mai, Pahang River | 1 | 3°47'34.33"N | 102°24'36.92"E |
| | 2 | 3°46'55.70"N | 102°24'13.37"E |
| | 3 | 3°46'28.45"N | 102°23'55.40"E |
| | 4 | 3°45'59.08"N | 102°24'7.62"E |
| | 5 | 3°45'31.79"N | 102°24'5.22"E |
| | 6 | 3°45'20.39"N | 102°23'37.79"E |
| | 7 | 3°45'37.26"N | 102°23'7.10"E |
| | 8 | 3°45'42.65"N | 102°22'27.27"E |
| | 9 | 3°45'34.68"N | 102°21'58.02"E |
| | 10 | 3°45'8.67"N | 102°21'57.02"E |
| | 11 | 3°44'40.24"N | 102°22'22.91"E |
| | 12 | 3°44'9.29"N | 102°22'26.80"E |
| Ulu Tembeling, Tembeling River | 1 | 4°26'53.34"N | 102°28'52.98"E |
| | 2 | 4°25'51.84"N | 102°27'25.16"E |
| | 3 | 4°25'40.47"N | 102°26'14.50"E |
| | 4 | 4°25'3.94"N | 102°26'8.66"E |
| | 5 | 4°24'40.63"N | 102°26'11.02"E |
| | 6 | 4°24'30.04"N | 102°26'1.51"E |
| | 7 | 4°26'47.37"N | 102°27'49.84"E |
| | 8 | 4°25'36.83"N | 102°26'50.26"E |

Those fish that could confidently be identified were enumerated and if still alive, were released back to their natural environment. Fish samples (about 1 to 5 samples) to represent each species were immediately preserved in 10% formalin, following 70% alcohol for deposition in Freshwater Fisheries Research Division, Fisheries Research Institute, Glami-Lemi, Jelebu, Negeri Sembilan. Collected fishes were identified to species level based on Mohsin and Ambak (1991), Zakaria-Ismail et al. (2019) and the latest scientific names and species validity followed Fricke et al. (2020).

During the fish sampling activities, the individual number of fish was recorded for each species, while the status of whether native or introduced fishes were also determined (Froese & Pauly, 2018; Zakaria-Ismail et al., 2019). The IUCN status of each collected fish species was determined using conservation assessment of the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species (International Union for Conservation of Nature's [IUCN], 2019).

**Water Physicochemical Analyses**

In triplicates, the water quality parameters were measured at the same site where the fish were sampled, specifically after deploying the fishing gears for fish capture. The measured parameters include water temperature (°C), pH (1-14), conductivity (µS/cm), total suspended solids (TSS) (mg/L) (YSI Incorporation, NY, USA), and river depth (m) (Speedtech Instrument, Logan, UT, USA). Besides that, the ammonia nitrogen ($NH_3$-N) (mg/L), phosphate ($PO_4^{3-}$) (mg/L), nitrate ($NO_3^-$) (mg/L) and alkalinity (mg/L) were measured using a spectrophotometer (HACH Company, Loveland, CO, USA).

**Statistical Analyses**

Fish diversity (Shannon-Wiener's diversity index (*H*) and Simpson's diversity index (*1-D*)), evenness (Pielou's evenness (*e*) index) and richness (Menhinnick's richness index (*I*)) indices for both rivers were enumerated. These were carried out using the PAST (version 2.15) software (Hammer et al., 2001). Using the same software, diversity t-tests of Shannon-Wiener's diversity index (*H*) and Simpson's diversity index (*1-D*) were carried out to ascertain if there exist significant differences in the fish species diversity of both sites. Furthermore, the temporal trends in the measured water physicochemical parameters were described and presented using graphs in Microsoft Excel (Office 365, Version 2016, Microsoft Corp., Berkshire, UK). Independent samples t-test was also used to compare water physicochemical parameters measured at both rivers using IBM SPSS, Version 22.0 (IBM Corp., Chicago, IL, USA). The significant difference was determined at $p < 0.05$. Prior to this, the data were log-transformed since they did not follow a normal distribution.

Data reduction was performed with Principal Component Analysis (PCA) to reduce variable numbers in the dataset by clustering highly correlated variables into factors while retaining variability in the

data with IBM SPSS, Version 22.0. By using PCA, the variables that correlated with one another were combined into factors. Canonical correspondence analysis (CCA) was thereafter used to determine the relationship between water quality and fish species assemblages in each river. The CCA was performed using XLSTAT add-in (Version 2014.5.3, Addinsoft, New York, NY, USA), with 1000 permutations at a significance level of 5%.

## RESULTS

### Fish Checklist, IUCN status, and Community Structures

A checklist of recorded fish species including their family name, scientific name, number of collected individuals, abbreviations, origin status, and IUCN status is presented in Table 2. This consists of 2,391 individuals, which were collected during this study, comprising of 20 families and 65 species of fish (not including *Pangasionodon* sp.). A total of 796 individuals belonging to 55 species from 17 families were collected from Kuala Mai, Pahang River. The highest represented family was Cyprinidae (22 species), followed by Bagridae, Pangasiidae and Siluridae with four species each. In Ulu Tembeling, Tembeling River, a total of 1,595 individuals belonging to 47 species and 15 families were collected. The family with the highest representation was Cyprinidae (22 species), followed by Danionidae with four species.

Of the 65 fish species recorded in this study, a total of seven species was considered as not evaluated (NE), four species as data deficient (DD), 52 species as least concern (LC), one species (*Bagarius yarrelli*) as near threatened (NT), one species (*Fluvitrygon signifier*) as endangered (EN), and one species (*Probarbus jullieni*) as critically endangered (CR). Furthermore, four introduced fish species such as *Barbonymus altus*, *Barbonymus gonionotus*, *Pangasionodon* sp. and *Pterygoplichthys disjunctivus* were also recorded in this study.

Fish community structures in Pahang and Tembeling rivers are presented in Table 3. Significant differences ($p < 0.05$) were only existed between the Shannon-Wiener's diversity (*H*) [($t(1799.4) = 9.234$, $p = 0.000$] and Simpson's diversity (*1-D*) ($t(2186.2) = -8.617$, $p = 0.000$] indices for both study sites, but not for evenness (*e* ) and richness (*D*) indices.

Table 2
*The family, species with number of individuals, IUCN status and abbreviation used of collected fishes in Kuala Mai, Pahang River, and Ulu Tembeling, Tembeling River*

| Family | Species | Pahang River | Tembeling River | IUCN status | Abbreviation |
|---|---|---|---|---|---|
| Dasyatidae | *Fluvitrygon signifier* | 1 | NA | EN | Hims |
| Anguillidae | *Anguilla marmorata* | NA | 1 | LC | Ang |
| Notopteridae | *Chitala lopis* | 2 | 6 | LC | Chil |
| | *Notopterus notopterus* | 4 | 4 | LC | Nop |
| Botiidae | *Syncrossus hymenophysa* | NA | 2 | LC | Synh |

Table 2 *(continue)*

| Family | Species | Pahang River | Tembeling River | IUCN status | Abbreviation |
|---|---|---|---|---|---|
| Cyprinidae | *Amblyrhynchichthys truncatus* | 23 | 66 | LC | Ambt |
| | *Barbichthys laevis* | 18 | 71 | LC | Barla |
| | *Barbodes lateristriga* | 1 | NA | LC | Barl |
| | **Barbonymus altus* | 1 | NA | LC | Hypn |
| | **Barbonymus gonionotus* | 3 | NA | LC | Barbd |
| | *Barbonymus schwanefeldii* | 88 | 130 | LC | Bars |
| | *Crossocheilus oblongus* | NA | 7 | LC | Cro |
| | *Cyclocheilichthys apogon* | 49 | 223 | LC | Cyca |
| | *Cyclocheilichthys repasson* | 16 | 11 | LC | Aner |
| | *Labeo chrysophekadion* | 2 | NA | LC | Labc |
| | *Labiobarbus festivus* | 57 | 104 | DD | Labf |
| | *Labiobarbus ocellatus* | NA | 5 | LC | Labo |
| | *Lobocheilos rhabdoura* | 23 | 160 | LC | Circ |
| | *Hampala macrolepidota* | 10 | 23 | LC | Ham |
| | *Hypsibarbus pierrei* | 3 | 3 | DD | Hypp |
| | *Hypsibarbus wetmorei* | 49 | 19 | LC | Hypw |
| | *Mystacoleucus obtusirostris* | 24 | 38 | NE | Myso |
| | *Osteochilus melanopleura* | 7 | 3 | LC | Osme |
| | *Osteochilus microcephalus* | 1 | 10 | LC | Osmi |
| | *Osteochilus spilurus* | NA | 25 | LC | Ospi |
| | *Osteochilus vittatus* | 23 | 10 | LC | Osvi |
| | *Osteochilus waandersii* | 24 | 2 | LC | Oswa |
| | *Probarbus jullieni* | 2 | 17 | CR | Proj |
| | *Puntioplites bulu* | 2 | 18 | LC | Pubu |
| | *Puntioplites proctozysron* | NA | 254 | LC | Punp |
| | *Tor tambra* | NA | 1 | DD | Tota |
| | *Thynnichthys thynnoides* | 43 | NA | LC | Thyn |
| Danionidae | *Rasbora dusonensis* | NA | 7 | NE | Bal |
| | *Rasbora elegans* | 18 | 48 | LC | Rasu |
| | *Luciosoma setigerum* | 12 | 9 | LC | Lucs |
| | *Raiamas guttatus* | 4 | 9 | LC | Ragu |
| Xenocyprididae | *Macrochirichthys macrochirus* | 6 | 5 | LC | Mac |
| | *Paralaubuca typus* | 28 | 12 | LC | Part |
| Ailiidae | *Laides hexanema* | 23 | 8 | LC | Lahe |
| Bagridae | *Bagrichthys macracanthus* | 2 | NA | LC | Bagm |
| | *Hemibagrus capitulum* | 21 | 49 | NE | Hemn |
| | *Hemibagrus wyckii* | 3 | 5 | LC | Hemw |
| | *Mystus castaneus* | 9 | 4 | LC | Mysg |
| Sisoridae | *Bagarius yarrelli* | 18 | 7 | NT | Bagy |

Table 2 *(continue)*

| Family | Species | Pahang River | Tembeling River | IUCN status | Abbreviation |
|---|---|---|---|---|---|
| Pangasiidae | *Helicophagus waandersii* | 5 | NA | LC | Hewa |
| | **Pangasionodon* sp. | 8 | NA | NE | Phy |
| | *Pangasius nasutus* | 10 | 8 | LC | Pana |
| | *Pseudolais micronemus* | 33 | 160 | DD | Psem |
| Siluridae | *Belodontichthys dinema* | 4 | 6 | LC | Beld |
| | *Kryptopterus limpok* | 7 | NA | LC | Kryl |
| | *Phalacronotus apogon* | 86 | 27 | LC | Phaa |
| | *Wallagonia leerii* | 2 | NA | LC | Wale |
| Loricariidae | **Pterygoplichthys disjunctivus* | 1 | NA | NE | Hple |
| Mastacembelidae | *Macrognathus maculatus* | 1 | 4 | LC | Mack |
| | *Mastacembelus erythrotaenia* | NA | 1 | LC | Mase |
| | *Mastacembelus favus* | 2 | 1 | LC | Masf |
| | *Mastacembelus unicolor* | 1 | NA | NE | Masu |
| Anabantidae | *Anabas testudineus* | 1 | NA | LC | Anat |
| Osphronemidae | *Osphronemus goramy* | 5 | 5 | LC | Ospg |
| | *Trichopodus trichopterus* | 6 | NA | LC | Tri |
| Channidae | *Channa lucius* | 1 | NA | LC | Chanl |
| | *Channa micropeltes* | NA | 5 | LC | Chanm |
| | *Channa striata* | 1 | NA | LC | Chans |
| Pristolepididae | *Pristolepis fasciata* | NA | 2 | LC | Prif |
| Soleidae | *Achiroides leucorhynchos* | 1 | NA | NE | Brpa |
| Belonidae | *Xenentodon canciloides* | 1 | NA | LC | Xenc |

(*) asterisk = indicates introduced species; numbers = number of individuals collected fish; NA = not available; NE = not evaluated; DD = data deficient; LC = least concern; NT = near threatened; EN = endangered; CR = critically endangered. The scientific names, species validity and species systematic order following California Academy of Science's Catalog of Fishes (Fricke et al., 2020)

Table 3
*Fish community structures in Kuala Mai, Pahang River and Ulu Tembeling, Tembeling River, based on diversity, evenness and richness indices*

| River | Community structure indices | | | |
|---|---|---|---|---|
| | *H* | *1-D* | *e* | *D* |
| Kuala Mai, Pahang River (n = 12) | 3.315 $\pm$ 0.036* | 0.949 $\pm$ 0.003* | 0.5 | 1.949 |
| Ulu Tembeling, Tembeling River (n = 8) | 2.893 $\pm$ 0.029 | 0.916 $\pm$ 0.003 | 0.384 | 1.177 |

*H* = Shannon-Wiener's diversity index; *1-D* = Simpson's diversity index; *e* = Pielou's evenness index; and *D* = Menhinnick's richness index; * = indicates a significant difference between both sampling sites

**Water Quality Measurements**

Figure 2 shows the trends in measured water quality parameters for Pahang and Tembeling rivers. In Kuala Mai, Pahang River, the river was deepest at 6.51 m in July and shallowest at 2.54 m in March, dropping from a depth of 6.03 m in January. The highest DO value of 8.33 mg/L was recorded in August, with the lowest value of 4.36 mg/L in September. Furthermore, pH ranged between 7.14 and 5.73 in January and November, while temperature ranged between 27.8°C, which was recorded in June and 25.24°C which was recorded in November. TSS was highest in August at 310.2 mg/L and lowest at 60.2 mg/L in October. During the study, $NH_3$-N ranged from a minimum of 0.3 mg/L in September, to a maximum of 1.7 mg/L in November. The greatest difference was observed between September and October. $NO_3^-$ ranged from 0.008 mg/L in February and 0.3 mg/L in September, with the greatest change observed between August and September. The highest $PO_4^{3-}$ content of 0.91 mg/L was observed in January and the lowest was 0.03 mg/L in August.

For Ulu Tembeling, Tembeling River, the river depth ranged from 4.83 m in April to 2.53 m in November. The highest DO value of 7.46 mg/L was recorded in August, with the lowest value of 6.23 mg/L recorded in March. pH ranged from 7.01 in November to 5.2 in October. Alkalinity was highest in October at 24.38 mg/L and lowest in June at 12.13 mg/L. Conductivity ranged from 98.88 µS/cm in November, and 28.00 µS/cm in the month of May. The highest temperature of 30.27°C was recorded in June, while the lowest at 25.81°C was observed in January. TSS ranged from 226.38 mg/L in October to 11.13 mg/L in the month of March. During the study period, $NH_3$-N ranged from 0.06 mg/L in November to 1.34 mg/L in October. $NO_3^-$ ranged from 0.02 mg/L in April to 0.21 mg/L in October. $PO_4^{3-}$ was at a minimum of 0.05 mg/L in June and a maximum of 1.20 mg/L in November.

Inferential statistical analyses revealed a significant difference ($p < 0.05$) between the two rivers for only three of the measured physicochemical parameters, such as temperature, $NH_3$-N and TSS (Table 4). Temperature ($t$ (16) = -2.171, $p = 0.045$) was significantly ($p < 0.05$) higher, while $NH_3$-N ($t$ (11.23) = 2.914, $p = 0.010$) and TSS ($t$ (13.469) = 4.167, $p = 0.010$) were significantly ($p < 0.05$) lower at Ulu Tembeling, Tembeling River compared to Kuala Mai, Pahang River. River depth, pH, alkalinity and $NO_3^-$ were numerically higher at Kuala Mai, Pahang River, while DO, conductivity, and $PO_4^{3-}$ were numerically lower at Kuala Mai, Pahang River.

**Relationships between Water Quality and Fish Occurrences**

Extraction and loadings from PCA of physicochemical parameters of Kuala Mai, Pahang River and Ulu Tembeling, Tembeling River are presented in Table 5. In each river, PCA generated two axes that cumulatively accounted for 81.01% and 84.13% variation in water physicochemical parameters for Kuala Mai, Pahang River, and Ulu Tembeling, Tembeling River, respectively.


*Figure 2*. Monthly trends in water physicochemical parameters. (a, b): ammonia nitrogen, phosphate, and nitrate; (c, d): dissolved oxygen, pH and depth; (e, f): conductivity and alkalinity, and; (g, h): total suspended solids and temperature for Kuala Mai, Pahang River (left column), and Ulu Tembeling, Tembeling River (right column)

Table 4
*Mean and range of water quality parameters for Kuala Mai, Pahang River, and Ulu Tembeling, Tembeling River*

| Variable | Kuala Mai, Pahang River (n = 12) | | Ulu Tembeling, Tembeling River (n = 8) | |
|---|---|---|---|---|
| | Mean ± SD | Range | Mean ± SD | Range |
| Depth (m) | 4.52 ± 1.24 | 2.50 - 6.51 | 3.80 ± 0.85 | 2.53 - 4.83 |
| Temperature (°C)* | 26.73 ± 0.80 | 25.24 - 27.80 | 28.22 ± 1.34 | 25.82 - 30.27 |
| DO (mg/L) | 6.72 ± 125.00 | 4.36 - 8.33 | 6.91 ± 0.40 | 6.23 - 7.46 |
| pH | 6.51 ± 0.39 | 5.73 - 7.14 | 6.33 ± 0.44 | 5.21 - 7.01 |
| Conductivity (µS/cm) | 46.07 ± 4.26 | 42.04 - 54.26 | 56.72 ± 24.32 | 28.00 - 98.88 |
| Alkalinity (mg/L) | 17.88 ± 3.70 | 10.60 - 22.20 | 16.35 ± 3.50 | 12.13 - 24.38 |
| $NH_3$-N (mg/L)* | 0.80 ± 0.43 | 0.30 - 1.73 | 0.39 ± 0.33 | 0.06 - 1.34 |
| $NO_3^-$ (mg/L) | 0.10 ± 0.07 | 0.01 - 0.26 | 0.07 ± 0.05 | 0.02 - 0.21 |
| $PO_4^{3-}$ (mg/L) | 0.27 ± 0.27 | 0.03 - 0.92 | 0.34 ± 0.37 | 0.05 - 1.20 |
| TSS (mg/L)* | 164.90 ± 89.73 | 60.20 - 310.60 | 48.71 ± 62.57 | 11.13 - 226.40 |

* = indicate significant difference ($p < 0.05$) on the mean of water quality parameters between both rivers

An eigenvalue of 1 or above was the benchmark for extraction. A minimum value of 1 is considered significant. A total of 10 physicochemical parameters were measured, and only six were retained for Kuala Mai, Pahang River, while five were retained for Ulu Tembeling, Tembeling River. In Kuala Mai, Pahang River, the first axis had strong loadings for DO, pH, and $PO_4^{3-}$, while the second axis indicated strong loadings for temperature, conductivity and alkalinity. Furthermore, at Ulu Tembeling, Tembeling River, the first axis had strong loadings for pH, alkalinity, $NH_3$-N and TSS, while the second axis was highly loaded only by $PO_4^{3-}$.

Six and five variables with significant contribution to the variation in the ordination resulting from the forward selection procedure of CCA were returned for Kuala Mai, Pahang River and Ulu Tembeling, Tembeling River, respectively (Table 6). The first and second ordination axes accounted for 24.39% and 21.13%, as well as 35.04% and 31.18% for Pahang and Tembeling rivers, respectively.

In Kuala Mai, Pahang River, based on the CCA ordination map, DO, followed by $PO_4^{3-}$, with the longest respective CCA vectors, were the most important water quality variables, while pH, conductivity and temperature presented much lower importance in structuring the fish occurrence, followed by alkalinity which was the lowest contributor among the six variables (Figure 3). Water quality parameters of pH and $PO_4^{3-}$ showed a negative correlation to alkalinity, while DO and temperature showed a negative correlation with conductivity. A total of 13 fish species including *Rasbora elegans*, *Hypsibarbus wetmorei*, *Raiamas guttatus*, *Lobocheilos rhabdoura*, *Fluvitrygon signifier*, *Channa striata*, *Bagrichthys macracanthus*, *B. altus*, *Macrognathus maculatus*, *Barbodes lateristriga*, *Amblyrhynchichthys truncatus*, *Osphronemus goramy*, *Phalacronotus*

*apogon*, and *Hemibagrus capitulum* were positively associated with higher $PO_4^{3-}$ and pH, but negatively associated with alkalinity levels, while the reverse was the case for other 15 species such as *Luciosoma setigerum*, *Achiroides leucorhynchos*, *Mystacoleucus obtusirostris*, *Chitala lopis*, *Wallagonia leerii*, *Hypsibarbus pierrei*, *Paralaubuca typus*, *Barbonymus schwanefeldii*, *Pangasius nasutus*, *Labeo chrysophekadion*, *Macrochirichthys macrochirus*, *Puntioplites bulu*, *Cyclocheilichthys repasson*, *Laides hexanema* and *P. jullieni* which were positively associated with alkalinity levels, but negatively associated with pH and phosphate levels. Meanwhile, 18 species such as *Kryptopterus limpok*, *Belodontichthys dinema*, *Channa lucius*, *Mastacembelus unicolor*, *Barbonymus gonionotus*, *Osteochilus waandersii*, *Cyclocheilichthys apogon*, *Mastacembelus favus*, *Hemibagrus wyckii*, *Anabas testudineus*, *Pterygoplichthys disjunctivus*, *Trichopodus trichopterus*, *Hampala macrolepidota*, *Osteochilus vittatus*, *B. yarrelli*, *Pseudolais micronemus*, *Notopterus notopterus*, and *Pangasionodon* sp. were negatively correlated with DO and temperature levels, but positively associated with conductivity. Conversely, species such as *Labiobarbus festivus*, *Barbichthys laevis*, *Helicophagus waandersii*, *Osteochilus melanopleura*, and *Thynnichthys thynnoides* were positively associated with DO and temperature levels but negatively associated with conductivity.

In Ulu Tembeling, Tembeling River, the CCA ordination map revealed that $PO_4^{3-}$, TSS and alkalinity were the most important physicochemical parameters, having the longest respective CCA vectors (Figure 4). $NH_3$-N concentration had less influence on the fish assemblages, while water pH

Table 5
*Loadings from the principal component analysis of water quality parameters in Kuala Mai, Pahang River and Ulu Tembeling, Tembeling River*

| Variable | Kuala Mai, Pahang River | | Ulu Tembeling, Tembeling River | |
|---|---|---|---|---|
| | PC1 | PC2 | PC1 | PC2 |
| Depth (m) | - | - | - | - |
| Temperature (°C) | -0.003 | 0.956* | - | - |
| DO (mg/L) | 0.880* | 0.019 | - | - |
| pH | 0.894* | -0.052 | -0.907* | 0.189 |
| Conductivity (µS/cm) | 0.320 | 0.888* | - | - |
| Alkalinity (mg/L) | -0.482 | 0.740* | 0.748* | 0.325 |
| $NH_3$-N (mg/L) | - | - | 0.922* | 0.081 |
| $NO_3^-$ (mg/L) | - | - | - | - |
| $PO_4^{3-}$ (mg/L) | -0.807* | -0.220 | 0.034 | 0.980* |
| TSS (mg/L) | - | - | 0.916* | 0.161 |
| Percentage variance explained | 42.66 | 38.35 | 61.46 | 22.67 |
| Cumulative variance explained | 42.66 | 81.01 | 61.46 | 84.13 |

* = Indicate strong loadings with absolute value (> 0.7)

Table 6
*Canonical Correspondence Analysis summary for water quality parameters in Kuala Mai, Pahang River and Ulu Tembeling, Tembeling River*

| | Kuala Mai, Pahang River | | | | | | Ulu Tembeling, Tembeling River | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | F1 | F2 | F3 | F4 | F5 | F6 | F1 | F2 | F3 | F4 | F5 |
| Eigenvalue | 0.272 | 0.236 | 0.196 | 0.174 | 0.124 | 0.114 | 0.189 | 0.168 | 0.086 | 0.060 | 0.037 |
| Cumulative % | 24.39 | 45.52 | 63.03 | 78.66 | 89.80 | 100.00 | 35.04 | 66.22 | 82.09 | 93.16 | 100.00 |
| Regression coefficients: | | | | | | | | | | | |
| pH | 0.426 | -0.644 | 1.969 | -1.536 | -0.084 | 0.966 | -0.063 | -0.179 | -0.941 | 0.229 | -0.163 |
| Alkalinity (mg/L) | -0.373 | 0.320 | -1.296 | -0.836 | -0.231 | -0.711 | -0.120 | 0.449 | 0.083 | -0.495 | -1.392 |
| $NH_3$-N (mg/L) | | | | | | | -0.916 | 0.419 | 0.443 | 1.937 | 0.382 |
| $PO_4^{3-}$ (mg/L) | 0.363 | -0.876 | 1.169 | -0.376 | -1.001 | 1.226 | -1.068 | 0.162 | -0.120 | -0.444 | 0.566 |
| TSS (mg/L) | | | | | | | 2.234 | 0.878 | -0.652 | -0.304 | 0.563 |
| Temperature (°C) | -0.439 | -0.315 | -1.734 | -0.564 | 0.905 | 1.067 | | | | | |
| DO (mg/L) | -1.039 | -0.364 | -2.343 | 1.034 | 0.086 | -1.357 | | | | | |
| Conductivity (µS/cm) | 0.498 | 0.063 | 4.508 | 0.145 | -1.427 | 1.176 | | | | | |

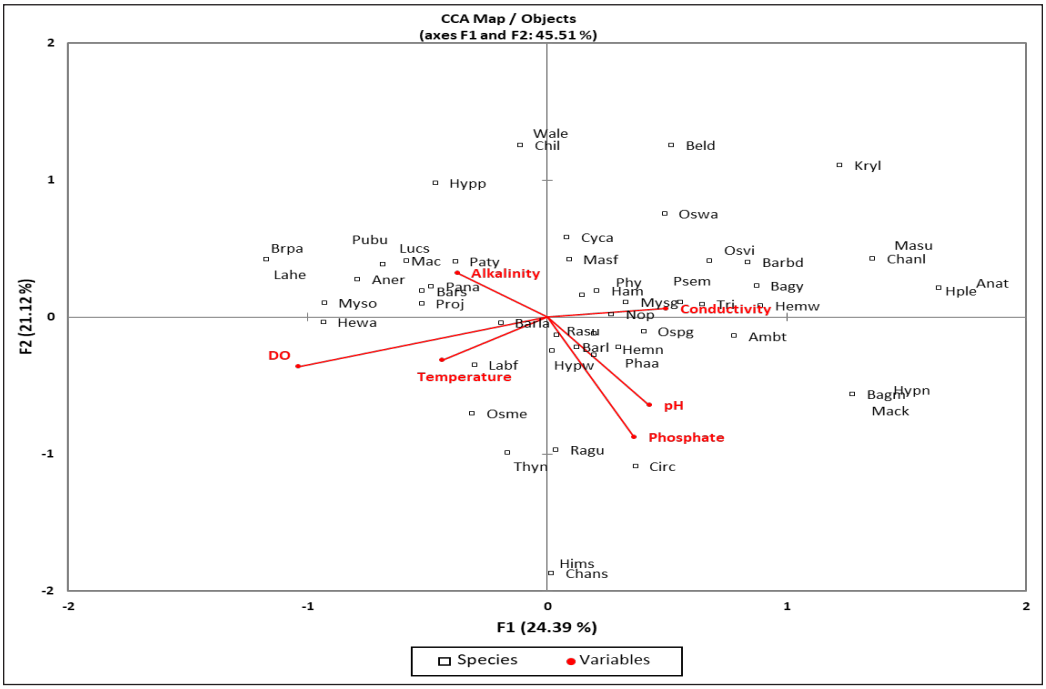


*Figure 3*. Canonical Correspondence Analysis ordination diagram showing the effect of environmental variables on fish assemblages in Kuala Mai, Pahang River

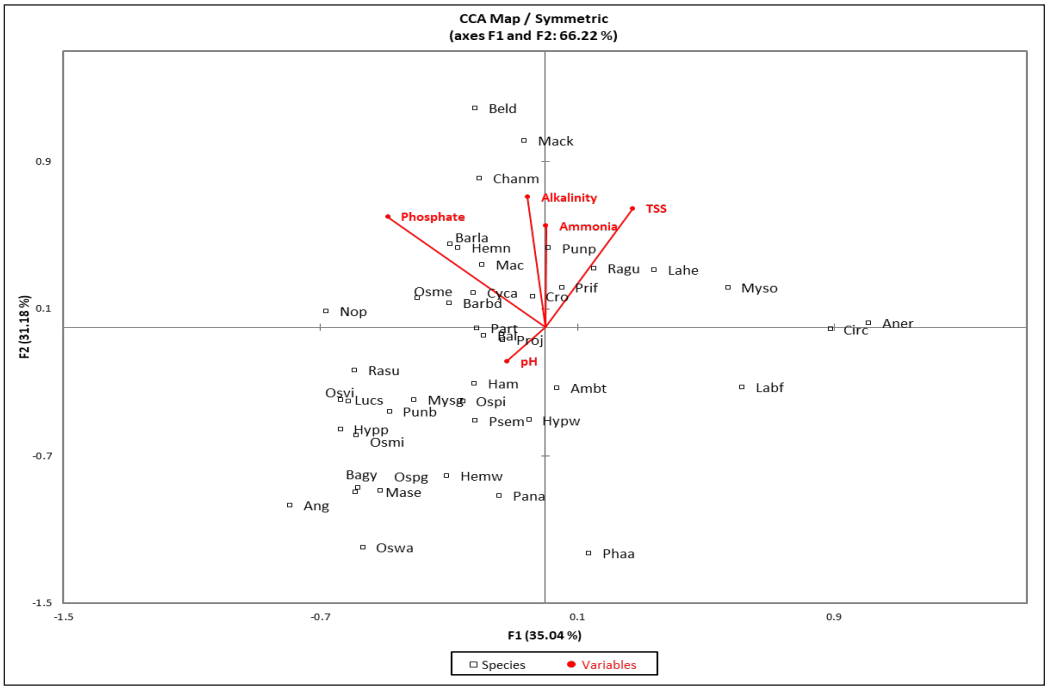


*Figure 4*. Canonical Correspondence Analysis ordination diagram showing the effect of environmental variables on fish assemblages in Ulu Tembeling, Tembeling River

correlated negatively with TSS. A total of 11 fish species such as *B. dinema*, *M. maculatus*, *Channa micropeltes*, *B. laevis*, *H. capitulum*, *M. macrochirus*, *O. melanopleura*, *C. apogon*, *B. gonionotus*, and *N. notopterus* correlated positively with $PO_4^{3-}$ and alkalinity, while three species including *A. truncatus*, *L. festivus*, and *P. apogon* correlated negatively with them. Nineteen species such as *R. elegans*, *O. vittatus*, *L. chrysophekadion*, *Puntioplites bulu*, *Mystus castaneus*, *Osteochilus spilurus*, *Pseudolais micronemus*, *Osteochilus microcephalus*, *B. yarrelli*, *O. goramy*, *Mastacembelus erythrotaenia*, *Anguilla marmorata*, *O. waandersii*, *Pangasius nasutus*, *H. wyckii*, *Paralaubuca typus*, *Probarbus jullieni*, and *Rasbora dusonensis* correlated positively with pH and negatively with TSS. Finally, six species such as *Puntioplites proctozysron*, *R. guttatus*, *L. hexanema*, *M. obtusirostris*, *C. repasson*, and *Pristolepis fasciata* correlated positively with TSS and negatively with pH levels.

## DISCUSSION

In this study, a total of 2,391 individuals that comprised of 65 fish species and 20 families were recorded for both rivers. Nevertheless, with reference to an earlier study carried out at Pahang River in the Maran District, a total of 2,075 individuals belonging to 65 species from 22 different families were recorded (Zulkafli et al., 2018), and a total of 4,834 fish individuals from 82 species and 25 families were collected from different parts of the Pahang River system, including Tembeling River (Zulkafli et al., 2015a). These findings indicate that Pahang and Tembeling rivers harbor high fish species diversity and strengthen the facts on their importance to the local community in terms of food production and income generation (Mutrimah et al., 2015; Zulkafli et al., 2018).

Out of 65 species recorded, only three species namely *B. yarrelli*, *F. signifier*, and *P. jullieni* have been identified in this study as near threatened, endangered and critically endangred, respectively (IUCN, 2019). However, the numbers of these collected individuals were very low (mostly one individual, respectively) compared to those identified as least concern and data deficient. This signals a need for more action in the protection of biodiversity of Pahang River and its tributaries. The above-listed species should receive more attention to avoid eventual extinction. Furthermore, four introduced fish species such as *B. altus*, *B. gonionotus*, *Pangasionodon* sp., and *P. disjunctivus* were also recorded in this study, where all of them were collected from Pahang River. The occurrence *B. gonionotus* and *Pangasionodon* sp. could be related to extensive aquaculture activities in Pahang River, while it was also good news as no introduced fish species observed in Tembeling River as it specifically flows along the National Park at Kuala Tahan, Pahang.

The diversity, evenness and richness indices reported in this study for Kuala Mai, Pahang River are higher than that reported previously in other parts of Pahang River by Zulkafli et al. (2015a). They reported Shannon-Wiener's diversity indices of 2.5,

2.7 and 2.4, and Pielou's evenness indices of 0.79, 0.88 and 0.78 for Maran, Temerloh and Jerantut Districts, respectively. In this study, although the number of fishes that were sampled from Ulu Tembeling, Tembeling River is higher compared to Kuala Mai, Pahang River, the Pahang River demonstrated higher diversity, richness, and evenness, indicating generally higher fish biodiversity. It is important to note that there was a difference in the sampling frequency which could have affected the fishing effort deployed at both rivers leading to difference in the diversity indices. Furthermore, a difference in river size, flow and area may influence the number of fishes caught (Santos et al., 2010).

Some water quality parameters such as temperature, $NH_3$-N and TSS were significantly different between the two sites, and the ranges recorded for $NO_3^-$ and $PO_4^{3-}$ were considered high. This situation may be a result of the monsoon season which occurs at east coast area at Peninsular Malaysia between the months of November and March, at times leading to heavy floods like that of 2006 to 2008, and this coincides with the sampling period for this study (Alias et al., 2016). In a study of the impacts of monsoon and dry seasons on physical water quality changes and farmed *Lates calcarifer* mortality at Sri Tujuh lagoon, Tumpat, Kelantan, Malaysia, it was concluded that the monsoon season influences certain water quality parameters (Abdullah et al., 2018). According to Abdullah et al. (2018), water quality parameters like DO and turbidity fluctuated and deteriorated during the monsoon season. The most crucial water physicochemical parameters influencing the occurrences of fishes in the Pahang River of Maran District include pH, temperature, conductivity, and $PO_4^{3-}$ (Zulkafli et al., 2018). For both study sites in this study, $PO_4^{3-}$ concentration was strongly related to the fish abundance, making it generally the most important physicochemical parameter as far as this study is concerned. Although alkalinity also recurred in both, it had less impact compared to that exhibited by $PO_4^{3-}$ concentration. Being a nutrient, the presence of $PO_4^{3-}$ may have likely influenced by primary production of food in the studied water bodies. On the other hand, if there is over enrichment in the water body, eutrophication may result leading to suboptimal water conditions. The less hardy fish species may move towards other regions with normal levels of $PO_4^{3-}$ (Nijboer & Verdonschot, 2004).

Temperature plays a crucial role in structuring fish occurrence. The physiology of fish can be affected by the temperature which is considered as the fundamental ecological variable (Norin et al., 2016). In Pahang River, the temperature was higher during the wet season, compared to the dry season. A study that had been done in the Three Gorges Reservoir, China and Pararanga Reservoir, Brazil, proved the water temperature might increase during the wet season (Deng et al., 2016). The reason for this higher wet seasonal temperature is not so clear because numerous factors such as hydrological including the relative contribution of groundwater, discharge

or flow rate, water volume, inflow from tributaries, climatological including air temperature, solar radiation, cloud cover, wind speed, vapor pressure, precipitation and evaporation (Dallas, 2008). Furthermore, aquatic organisms' reaction towards the other biotic and abiotic stressors can be affected by thermal stress on fish (Grasset et al., 2016). In Pahang River, $PO_4^{3-}$ and DO were the most important predictors of fish occurrence. Although water conductivity had more influence on fish occurrences in Kuala Mai, Pahang River compared to Ulu Tembeling, Tembeling River, the possible influence seems to be weak compared to other physicochemical parameters influencing that river.

For Ulu Tembeling, Tembeling River, some fish species seem to prefer high pH areas with a low concentration of TSS, alkalinity, and $NH_3$-N. This group was dominated by various kinds of fish families, especially Pangasiidae and Bagridae. The pH reading of 5 to 6 is considered natural acidity which is produced by organic acids, and it has been mentioned that natural acidity from organic acids has less effect on constructing fish assemblages compared to the acidity that comes from human impacts (Greig et al., 2010). In addition, the pH may not be easily predictable because of the possible influence of dissolved leachate during the wet season. This may increase the water acidity as opposed to the expectation of increased pH with a higher influx of water (Nienie et al., 2017).

The patterns of the fish assemblage were also influenced by TSS. Fewer species were found in the area with a high value of TSS. For instance, *L. hexanema* would prefer and could live in the area with low pH and high concentration of TSS. Moreover, abiotic factors such as water temperature, pH and TSS had a significant effect on the fish survival rate, while fish survival based on suspended solids still differ across species depending upon species-specific tolerance to suspended solids (Nyanti et al., 2018).

## CONCLUSION

Useful background information for the management of Pahang and Tembeling rivers through knowledge of the diversity, dynamics of water physicochemical parameters, and interaction between fish species occurrences and water quality parameters were presented in this study. Apart from the influences of $PO_4^{3-}$, alkalinity and pH in both rivers, the results showed that temperature, DO, and conductivity were the major influencers of the fish occurrences in Kuala Mai, Pahang River, as $NH_3$-N, and TSS in Ulu Tembeling, Tembeling River. Information on the dynamics of these important parameters can help in the monitoring of water quality, and possibly in predicting the abundance of certain fish species of economic and ecological importance. The occurrence of introduced fish species in the natural water body of the Pahang River should raise some concerns to the authorities. Moreover, fish conservation and stock management efforts are urgently needed due to the decreasing number of near-threatened, endangered and critically endangered fish species in both rivers.

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# RAPD Analysis of *Musa acuminata* cv. Berangan Plantlets in Nursery Stage from Long-term Subculture

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## ABSTRACT

Banana *Musa acuminata* cv. Berangan is an important fruit crop in Malaysia. The use of tissue culture techniques can increase the number of planting materials for mass production of banana. However, the main problem in banana tissue culture is somaclonal variation, which is caused by many factors, such as long-term subcultures, which can reduce the production value and quality. In this study, the experiment focused on somaclonal variation caused by long-term subculture that had caused changes in their morphology which could be differentiated by RAPD pattern of micropropagated *Musa acuminata* cv. Berangan plantlets from long-term subcultures (15$^{th}$ subculture). Banana plantlets established from micropropagated banana using MS supplemented with 5 mgL$^{-1}$ of BAP was maintained until 15$^{th}$ subculture before being hardened and acclimatized in commercial soils. In this experiment, banana seedlings were categorized into four groups based on their heights at 5 - 10 cm, 11 - 15 cm, 16 - 20 cm and 21 - 25 cm. Results showed that the tallest seedlings (21 - 25 cm) produced 9.56 ± 1.01 number of leaves, 218.88 ± 40.89 cm leaf area and 5.32 ± 0.78 cm girth of pseudostem whereby the shortest group (5 - 10 cm) produced 5.67 ± 0.98 leaves, 18.95 ± 12.37 cm of leaf area, and 1.63 ± 0.54 cm girth of pseudostem. RAPD analysis carried out using two primers, OPH09 and OPA15 showed variation between the tallest seedlings and the shortest seedlings. This study concluded that long-term subculture of banana cv. Berangan produced variation in the seedlings' growth thus may affect the quality of planting materials produced.

*Keywords*: Banana, Berangan, micropropagated, plant height, RAP

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## INTRODUCTION

Banana is the first fruit crop to adopt tissue culture technique for mass propagation and is still actively used for commercial production compared to other fruit crops (Swennen et al., 2004). Conventional banana propagation using suckers has limitations for large-scale planting because only 5 to 10 suckers per clump can be produced per year (Rahman et al., 2002). Moreover, diseases can spread quickly through vegetative propagation as it may carry source of pathogen from soil, whereas tissue culture can rapidly produce healthy planting materials on a large scale (Kaçar & Faber, 2012). In the field, tissue cultured banana seedlings show better agronomical characteristic compared to suckers (Bairwa et al., 2015; Mensah et al., 2012). After going through the *in vitro* process for multiplication, plantlets must undergo acclimatization stage. Acclimatization takes place to allow plantlets to adapt to new environment which involve physiological adaptation due to changes in surrounding condition such as temperature, light and humidity in the nursery (Perez & Hooks, 2008; Scaranari et al., 2009). Acclimatization of banana tissue cultured plantlets in the soil is a critical stage as they are subjected to different stresses that may affect their survival as they need to adapt to new environment (Aragón et al., 2005; Elisama et al., 2013; Vasane & Kothari, 2006). Lower relative humidity and higher light exposure can cause wilting and necrosis of leaves which eventually may result into failure to survive during the acclimatization phase (Preece & Sutter, 1991).

Good management and planning of acclimatization phase are a combination of morphological, biochemical and physiological processes to produce better quality plantlets which can survive in the field (Bitar & Mohamed, 2009; Vasane & Kothari, 2008). In glasshouse, higher irradiance and lower humidity can affect the survival rate (Kaçar & Faber, 2012; Vasane & Kothari, 2006) as they usually need some weeks for acclimatization (Vasane & Kothari, 2008). Many seedlings die and wilting due to water loss in the leaves which is often not restricted during the first transplant to the soil as there are still poor conductivity of roots and root-stem connections. Therefore, there is a need to limit the water supply to reduce hydraulic conductivity between roots and stems (Kaçar & Faber, 2012). Reducing natural light by using shading is essential during the acclimatization process (Scaranari et al., 2009). Successful acclimatization of plantlets depends on the ability for high survival rates (Zaid & Hughes, 1995). Carbohydrates content such as starch and sucrose plays an important role in the acclimatization process (Aragón et al., 2005, 2006). Many studies on the acclimatization of banana tissue cultures to the soils have been reported (Choudhary et al., 2014; Elhory et al., 2009; Husin et al., 2014).

The profit gain in banana tissue culture industry depends on the multiplication rate

in every number of subculture (Chavan-Patil et al., 2010). However, Sheidai et al. (2008) reported that a high number of subculturing might produce a few percentages of somaclonal variation in the banana tissue culture. This was supported by Borse et al. (2011), who stated that higher multiplication rates could also produce more variation. Mohamed (2007) suggested that somaclonal variation rate was much higher after the 6th clonal generation. Martin et al. (2007) stated that deficiency of calcium due to necrosis would occur after the 7th subculture for the banana cv. Cavendish. Furthermore, better yield was produced from the 8th subculture as compared to the 15th subculture (Chavan-Patil et al., 2010). Seven months of culturing in solid medium media can trigger genetic changes (Aremu et al., 2013; El-Dougdoug et al., 2007) and variation can be increased with frequent number of subcultures (Rodrigues et al., 1998; Sheidai et al., 2008). However, Lakshmanan et al. (2007) reported that there were no genetic changes in a number of subcultures up to 150 times for banana cv. Nanjanagudu Rasabale.

Molecular marker technology such as random amplified polymorphism DNA (RAPD) is a very useful technique to discover polymorphism in the DNA sequence for analysis of genetic variations, such as somaclonal variations (Chinmayee et al., 2012). The study was conducted to identify morphology and genetic changes in the long-term micropropagated banana cv. Berangan plantlets using RAPD.

## MATERIALS AND METHODS

### Plant Material

Banana cv. Berangan plantlets raised *in vitro* up to 15th of subculture using MS medium supplemented with 5 $mgL^{-1}$ of BAP in plant tissue culture laboratory of Universiti Malaysia Kelantan were acclimatized using commercial soil in a nursery. After 3 months of acclimatization, the performance of each seedlings was recorded. Morphological parameters observed were plant height (measurement was taken from the point above the surface, up to the point at the last intersection of two new leaves), number of leaves, leaf length (determined by measuring of the second leaf), leaf width (determined by measuring the second leaf) and girth of pseudostem (measured at 1 cm above ground level using a tape-meter). Measurement for these parameters were as shown in Figure 1. Leaf area was calculated

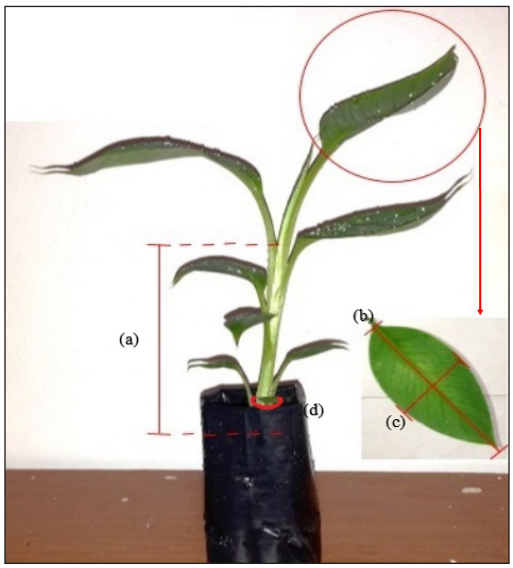


*Figure 1*. Photo showing measurement for (a) plant height, (b) leaf length of the 2nd leaf, (c) leaf width of the 2nd leaf, and (d) girth of the pseudostem

using equation following Turner and Lahav (1983). DNA extracted from banana leaves of different treatments were subjected to RAPD analysis.

### DNA Extraction

Total genomic DNA was extracted using CTAB method from young leaf samples following the protocol by Doyle and Doyle (1987). DNA qualification and quantification were determined and samples were diluted to a concentration of 25 ng $\mu L^{-1}$.

### Random Amplified Polymorphism DNA Analysis

In this experiment, DNA from different groups was pooled from 10 replications. The experimental design for this study consisted of only two groups of the highest and lowest plants heights (groups 1 and 4) which were subjected to RAPD analysis. RAPD analysis was performed following the method proposed by Weising et al. (1995). DNA amplification reactions were prepared for 25 µl reaction containing 50 ng $\mu l^{-1}$ of banana genomic DNA, 1× PCR buffer, 200 µM of dNTPs, 2.5 mM $MgCl_2$, 10 pmoles of 7 random decamer oligonucleotide primers from different sets (OPA19, OPJ04, OPA06, OPH09, OPA01, OPA02 and OPA15) and 1.25 U *Taq* DNA polymerase. Amplification reactions were carried out using an Eppendorf Thermal Cycler at 95ºC for 3 min for initial denaturation. This was followed by 40 cycles at 30 sec denaturation kept at 95ºC, primer annealing at 36ºC for 30 secs, primer extension for 2 min at 72ºC, and a final extension at 72ºC for 5 min. This was then held at 4ºC. 100 bp DNA ladders was used as standard markers and PCR products were subjected to electrophoresis using 1X TBE buffer consisting of tris base at 10.8 $gL^{-1}$, boric acid ($H_3BO_3$) at 5.5 $gL^{-1}$, EDTA at 0.74 $gL^{-1}$ and pH 8 in 1.4% (w/v) agarose gel. Gels were stained with 0.0001% ethidium bromide and visualized under UV and photographed.

## RESULTS AND DISCUSSION

After three months of acclimatization (Figures 2 and 3), the plant height increased significantly in all four different groups on the plant height at 6.71 ± 2.01 cm, 13.55 ± 1.18 cm, 17.13 ± 1.11 cm and 21.74 ± 1.69 cm, respectively (Table 1). The number of leaves of different groups also

Table 1
*Plant height, number of leaves, leaf area and girth of pseudostem for* Musa acuminata *cv. Berangan after 3 months being acclimatized in the nursery*

| Group of seedling (cm) | Plant height (cm) | No. of leaves | Leaf area ($cm^2$) | Girth of pseudostem (cm) |
|---|---|---|---|---|
| 5-10 | $6.71 \pm 2.01^{d}$ | $5.67 \pm 0.98^{c}$ | $18.95 \pm 12.37^{d}$ | $1.63 \pm 0.54^{d}$ |
| 11-15 | $13.55 \pm 1.18^{c}$ | $8.57 \pm 0.90^{b}$ | $87.01 \pm 31.29^{c}$ | $3.56 \pm 0.60^{c}$ |
| 16-20 | $17.13 \pm 1.11^{b}$ | $9.38 \pm 0.79^{a}$ | $156.98 \pm 37.94^{b}$ | $4.40 \pm 0.35^{b}$ |
| 21-25 | $21.74 \pm 1.69^{a}$ | $9.56 \pm 1.01^{a}$ | $218.88 \pm 40.89^{a}$ | $5.32 \pm 0.78^{a}$ |

*Note.* Different letters indicate values are significantly different ($P \leq 0.05$) by Duncan's multiple range test; Values are mean ± standard deviations based on at least ten replicates

increased at 5.67 ± 0.98 cm, 8.57 ± 0.90 cm, 9.38 ± 0.79 cm and 9.56 ± 1.01 cm, respectively (Table 1). However, only two groups showed a significant increment in the number of leaves at 5.67 ± 0.98 cm for the 5 - 10 cm group and 8.57 ± 0.90 cm for the 11 – 15 cm group while the remaining two groups were not showing significant result. Meanwhile the leaf area for the 2nd leaf was highly significant at 18.95 ± 12.37 cm, 87.01 ± 31.29 cm, 156.98 ± 37.94 cm and 218.88 ± 40.89 cm, respectively. The girth of pseudostem of the banana seedlings also showed significant increment at 1.63 ± 0.54 cm, 3.56 ± 0.60 cm, 4.40 ± 0.35 cm and 5.32 ± 0.78 cm, respectively.

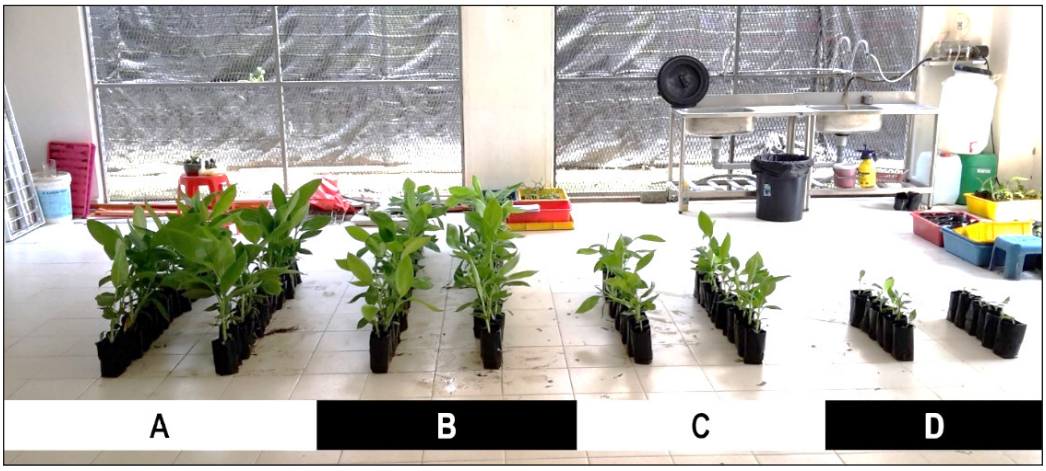


*Figure 2*. Four groups of long-term micropropagation of banana cv. Berangan plantlets based on different plant height observed after three months of acclimatization. A: 20 - 25 cm, B: 16 – 20 cm, C: 11 - 15 cm, and D: 6 – 10 cm


*Figure 3*. Morphology of banana cv. Berangan seedlings across different groups based on plant height (1) 20 - 25 cm, (2) 16 – 20 cm, (3) 11 - 15 cm, and (4) 6 – 10 cm after 3 months being acclimatized in the nursery

Plantlets produced *in vitro* must adapt to nursery condition before being transplanted into the field (Aragón et al., 2006). This is a critical stage whereby the banana plantlets need to undergo acclimatization process as they will be subjected to different environments as well as physiological and biochemical changes during this period (Pati et al., 2013). The use of polybags helps to deliver the greatest development of tissue-cultured banana plantlets as it provides the best draining system that does not allow roots to drown with over supply of water (Perez & Hooks, 2008). Banana cv. Grand Naine, Amritasagar and Sabri plantlets had in the past successfully survived after acclimatization using micropropagated banana from MS medium supplemented with 5 $mgL^{-1}$ of BAP in the nursery (Hossain et al., 2016). Plant height of the cultivated banana seedlings obtained after acclimatization depends on variety and their environments. Study by Manjula et al. (2014) showed that maximum shoot height observed for banana cv. Rajapuri was 16.17 cm from plantlets cultured in MS medium supplemented with 5 $mgL^{-1}$ of BAP and 2 $mgL^{-1}$ of NAA after 75 days acclimatized in the nursery. They noticed that 20 % of plantlets cultured in MS medium supplemented with 0.2 $mgL^{-1}$ TDZ produced dwarfism. However, the highest plant height obtained for banana cv. Grand Naine in the nursery after 45 days of acclimatization was 23.6 cm (Vasane & Kothari, 2008) and 24.3 cm (Vasane & Kothari, 2006). Banana seedlings with the height of around 20 cm were suitable for transferring in the field (Perez & Hooks, 2008). Banana seedling height of 15.7 cm for cv. Grand Naine produced 8.66 number of leaves after 45 days of acclimatization in the nursery (Ahmed et al., 2014). In addition, banana seedlings of 13.89 cm in height produced 4.7 number of leaves for cv. Nanjanagud Rasabale after 45 days in the nursery (Waman et al., 2014) while banana cv. Shima produced 7.5 number of leaves at 50 cm height after two months in the nursery (Buah et al., 2000).

Genetic differences between groups 1 and 4 were evaluated using RAPD analysis (Figure 4). In this study, two of seven RAPD primers (OPH09 and OPA02) showed loss of bands when compared between the two groups of different plant heights described in Figure 4. Primer OPH09 produced four bands in group 1 while five bands were observed in group 4. However, primer OPA15 lost 3 bands in the tallest group of plantlets as compared to the shortest group. Figure 4 also showed that primers OPH09 and OPA02 also displayed polymorphism. Primer OPH09 showed a unique 1800 bp fragment in group 4 which was absent in group 1. In addition, the RAPD profile produced by primer OPA02 had a missing band at 1700 bp in group 1 but present in group 4. Long-term subculture was the main focus in this study in order to detect genetic differences using the RAPD analysis. Results showed that RAPD primers could distinguish between groups of 1 and 4. The banana seedlings morphology of group 1 was considered as normal plant height as compared to group 4, which was depicted by a dwarf morphology. This morphology

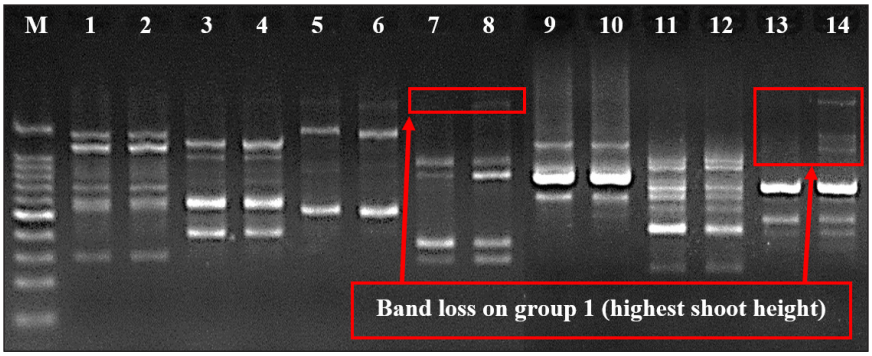


*Figure 4*. RAPD profiles of different groups of banana cv. Berangan seedlings after 3 months acclimatized in nursery conditions based on their height (groups 1 and 4) of in (lanes 1, 3, 5, 7, 9, 11 and 13) tallest group of plant height and (lanes 2, 4, 6, 8, 10, 12 and 14) group 4 with shortest plant height [RAPD profiles using decamer primers (lanes 1 and 2) OPA19, (lanes 3 and 4) OPJ04, (lanes 5 and 6) OPA06, (lanes 7 and 8) OPH09, (lanes 9 and 10) OPA01, (lanes 11 and 12) OPA02 and (lanes 13 and 14) OPA15. M: 100kb DNA ladder]

resulted from the effects of long-term exposure to *in vitro* condition (up to 15$^{th}$ subculture) which had led to genetic changes that could be determined by using RAPD analysis.

The use of RAPD analysis for nursery studies had been conducted by several researchers for detection of somaclonal variations in banana tissue cultures (Chinmayee et al., 2012; Ganapathi et al., 2008). However, study by Chinmayee et al. (2012) showed that clones of different traits consisting of normal, dwarf and giant at nursery stage showed different banding patterns when analyzed using RAPD.

## CONCLUSION

Micropropagated banana cv. Berangan from long-term subculture produced different plant heights ranging from 5 to 25 cm which was not uniform. This phenomenon is related to the genetic changes which can be proven by using RAPD analysis. RAPD analysis showed that banana cv. Berangan plantlet height of 20 – 25 cm depicted variation as compared to 6 – 10 cm plantlets.

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# Effect of High Temperature and Light Intensity on Physiology and Morphology in Young *Dipterocarpus alatus* Roxb. Leaf

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## ABSTRACT

Heat and high light intensity affected physiology and morphology of young *Dipterocarpus alatus* Roxb. leaf studied. *D. alatus* is a native forest tree and being extended to cultivation in the field as an economic crop. Nowadays, climate change due to increasing in temperature and light intensity can affect growth, morphological and photosynthetic traits in *D. alatus*. This research aimed to study the effects of high temperature and strong light intensity on physiology and morphology of the young *D. alatus*. The experiment was decided in CRD with 5 replications. The two-year-old *D. alatus* was treated with combination stress between temperature (at 35°C or 41°C) and light intensity (at 700 or 1800 µmol $m^{-2}s^{-1}$) for 7 days. Plant morphology, gas exchange, PSII efficiency and photosynthetic pigment contents were measured. Strong light intensity (1800 µmol $m^{-2}s^{-1}$) affected plant morphology by leaf burning and heat injury. However, high temperature (41°C) combined with strong irradiation enlarged leaf injury and also increased percentage of heat injury (3.01±0.81%; T41L1800) compared to control (0.07±0.00%; T35L700). In contrast, it reduced percentages of leaf angle (-8.77±2.82%) and leaf area (-1.04±0.38%). In addition, the combination stress influenced reduction of net photosynthetic rate and contents of Chl *a*+*b* and Chl *a* but unaffected Chl *b* and *Car* contents. Therefore, combined stress affected young *D. alatus* by damaging

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photosynthetic pigments such as Chl *a* and injured leaf tissue. This resulted in reduction in both of photosynthetic mechanism and *D. alatus* leaf growth. Thus, young *D. alatus* leaf (two-year-old) was susceptible to heat combined with excessive light.

*Keywords*: Climate change, *Dipterocarpus species*, gas exchange, photosynthetic pigment

## INTRODUCTION

*Dipterocarpus alatus* is a large tree species found in native tropical forest. Presently, heat and excessive light are harmful to global climate, thus there is an extension of *D. alatus* planting in the field as an economic crop for increasing green areas and income of farmers. In 2013, there were only a few forest areas in Thailand (approximately 31.58%) ("Forest area of Thailand", 2016). This causes global warming by increasing in temperature and strong irradiation. The prediction is global temperature in 2081 - 2100 rises up in the range 2.6 - 4.8°C (Intergovernmental Panel on Climate Change [IPCC], 2014). The global warming to 1.5°C increases the risk for long-term to change ecosystem, it can be explained by increases sea-level (IPCC, 2018). High temperature causes thylakoid membrane to lose its function and inner membrane of chloroplast is compromised causing decreases in chlorophyll content (Xu et al., 1995). At the temperature above 35°C, PSII efficiency was found decreased (Sanchez-Reinoso et al., 2014). When the plants are exposed to non-optimum temperature, the overall photosynthesis including PSII efficiency shows reduction. In addition, in tree, high temperature affects its growth and production by reducing photosynthesis. The photosynthetic response and tree recovery capacity under heat stress were investigated by gas exchange, chlorophyll fluorescence and electron transport. These measurements found that photosynthesis showed complete recovery after being treated with high temperature for less than 6 hr (Song et al., 2014). Vannajan (1997) reported that in *D. alatus* after being exposed to different temperature (18, 24, 30 and 36°C) and different Photosynthetic Photon Flux Density (PPFD) (0, 500, 1,000, 1,500 and 2,000 μmol $m^{-2}s^{-1}$) showed the values of net $CO_2$ gas exchange decreased with increasing in PPFD. In addition, transpiration rate increased but maximum net photosynthetic rate decreased at low temperature. Light intensity also affects plant growth and development and partitioning which are directly related to plant photosynthesis and metabolism. Plants exposed to strong light results in leaf burn and plant growth inhibition. *Dipterocarpus alatus* seedlings after being treated with light intensity at 10, 30, 50 and 100% showed that growth rate of their seedlings exposed to light intensity at 30 and 100% was similar. The optimum light intensity of *D. alatus* seedlings after transplanting was 50%. This resulted in higher survival and growth rate than other light intensities. In dry season, *D. alatus* seedlings exposed to 30% of light intensity showed higher in growth rate and strength of seedling than at 50 and 100 % (Bupabanpot et al., 1991). In addition, the high temperature and high

light intensity also influences to other plant species. Donsansuk et al. (2017) reported that in rice cv. PT60 after short-term heat exposure (40°C, 30 min) showed a decline in PSII efficiency and chlorophyll contents. Jagadish et al. (2007) found that in rice after exposure to high temperature above 33.7°C for less than 1 hr resulted in produced sterile seeds. Therefore, this research aimed to study the effect of high temperature and strong irradiation on photosynthetic performance, photosynthetic pigments and morphology of young *D. alatus* for basic knowledge.

## MATERIALS AND METHODS

### Plant Materials and Experimental Conditions

Two-year-old of *D. alatus* seedling was provided by Khon Kaen Plant Cultivation Center. *Dipterocarpus alatus* seedlings were transplanted in the pots (12 × 9 inch) containing mixed soil (loam : planting soil; 2 : 1) and planted in open air greenhouse at Agronomy field, Department of Agronomy, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand. The environmental conditions in greenhouse during April-December 2017 were shown as following: relative humidity = 88 - 96% and air temperature = 23.4 - 29.8°C. Then, these seedlings were treated with combination stress between temperature (at 35°C or 41°C) and light intensity (at 700 or 1800 µmol $m^{-2}s^{-1}$) in controlled temperature chamber (VRV. Corp., Ltd, Thailand).

### Temperature and Light Intensity Treatment

After transplanting, healthy seedlings were selected for exposure with temperature combined with light intensity. All combination treatments were carried out in controlled temperature chamber (VRV. Corp., Ltd, Thailand), Plant Physiology Laboratory, Department of Agronomy, Faculty of Agriculture, Khon Kaen University, Thailand. The combined treatments of temperature and light intensity are shown in Tables 1 to 4.

### Determination of Plant Growth and Morphology

Plant growth and morphology such as leaf number, plant height, leaf angle, leaf area and percentage of heat injury were investigated before (0 Day after treatment; DAT) and after (7 DAT) treated with combined conditions. Leaf number was counted in a whole plant. Plant height was measured from above soil ground to leaf tip of *D. alatus* seedlings. Leaf angle was manually measured in the $1^{st}$ – $6^{th}$ leaves from shoot tip by using protractor 180 degree (size = 4 inch). Six mature leaves were collected at 0 and 7 DAT for determining leaf area and percentage of heat injury. Both of their methods were investigated by taking a photo and analyzing them using Photoshop programmer (Adobe Photoshop CS6).

Table 1

*The combined treatment of temperature and light intensity in controlled temperature chamber (VRV. Corp., Ltd, Thailand) (T35L700) was set up at 35°C during 12.00 p.m. - 3.00 p.m. and at 700 µmol $m^{-2}s^{-1}$ during 7.00 a.m. - 6.00 p.m., respectively and relative humidity was in the ranged between 50 – 80% during 12.00 a.m. – 12.00 p.m.*

| Diurnal time (hr) | Temperature (°C) | Relative humidity (%) | Light intensity* (µmol $m^{-2}s^{-1}$) |
|---|---|---|---|
| 12.00 am - 3.00 a.m. | 28 | 65 | 0 |
| 3.00 a.m. - 7.00 a.m. | 25 | 80 | 0 |
| 7.00 a.m. - 9.00 a.m. | 27 | 70 | 700 |
| 9.00 a.m. - 10.00 a.m. | 30 | 60 | 700 |
| 10.00 a.m. - 12.00 p.m. | 33 | 55 | 700 |
| 12.00 p.m. - 3.00 p.m. | 35 | 50 | 700 |
| 3.00 p.m. - 5.00 p.m. | 33 | 55 | 700 |
| 5.00 p.m. - 6.00 p.m. | 32 | 56 | 700 |
| 6.00 p.m. - 9.00 p.m. | 30 | 60 | 0 |
| 9.00 p.m. - 12.00 a.m. | 28 | 65 | 0 |

*Note.* The temperature and relative humidity were derived from the statistic data of weather in Agronomy field, Faculty of Agriculture, Khon Kaen University, during March - May 2016 and 2017

*The distance of light intensity was 5 cm. far from the light source incident upper leaf

Table 2

*The combined treatment of temperature and light intensity in controlled temperature chamber (VRV. Corp., Ltd, Thailand) (T35L1800) was set up at 35°C during 12.00 p.m. - 3.00 p.m. and at 1800 µmol $m^{-2}s^{-1}$ during 10.00 a.m. – 3.00 p.m., respectively and relative humidity was in the ranged between 50 – 80% during 12.00 a.m. – 12.00 p.m.*

| Diurnal time (hr) | Temperature (°C) | Relative humidity (%) | Light intensity* (µmol $m^{-2}s^{-1}$) |
|---|---|---|---|
| 12.00 a.m. - 3.00 a.m. | 28 | 65 | 0 |
| 3.00 a.m. -7 .00 a.m. | 25 | 80 | 0 |
| 7.00 a.m. - 9.00 a.m. | 27 | 70 | 700 |
| 9.00 a.m. - 10.00 a.m. | 30 | 60 | 700 |
| 10.00 a.m. - 12.00 p.m. | 33 | 55 | 1800 |
| 12.00 p.m. - 3.00 p.m. | 35 | 50 | 1800 |
| 3.00 p.m. - 5.00 p.m. | 33 | 55 | 700 |
| 5.00 p.m. - 6.00 p.m. | 32 | 56 | 700 |
| 6.00 p.m. - 9.00 p.m. | 30 | 60 | 0 |
| 9.00 p.m. - 12.00 a.m. | 28 | 65 | 0 |

*Note.* The temperature and relative humidity were derived from the statistic data of weather in Agronomy field, Faculty of Agriculture, Khon Kaen University, during March - May 2016 and 2017

*The distance of light intensity was 5 cm. far from the light source incident upper leaf

Table 3
*The combined treatment of temperature and light intensity in controlled temperature chamber (VRV. Corp., Ltd, Thailand) (T41L700) was set up at 41°C during 12.00 p.m. - 3.00 p.m. and at 700 µmol $m^{-2}s^{-1}$ during 7.00 a.m. – 6.00 p.m., respectively and relative humidity was in the ranged between 40 – 80% during 12.00 a.m. – 12.00 p.m.*

| Diurnal time (hr) | Temperature (°C) | Relative humidity (%) | Light intensity* (µmol $m^{-2}s^{-1}$) |
|---|---|---|---|
| 12.00 a.m. - 3.00 a.m. | 28 | 65 | 0 |
| 3.00 a.m. - 7.00 a.m. | 25 | 80 | 0 |
| 7.00 a.m. - 9.00 a.m. | 30 | 65 | 700 |
| 9.00 a.m. - 10.00 a.m. | 35 | 56 | 700 |
| 10.00 a.m. - 12.00 p.m. | 38 | 45 | 700 |
| 12.00 p.m. - 3.00 p.m. | 41 | 40 | 700 |
| 3.00 p.m. - 5.00 p.m. | 38 | 45 | 700 |
| 5.00 p.m. - 6.00 p.m. | 35 | 50 | 700 |
| 6.00 p.m. - 9.00 p.m. | 32 | 55 | 0 |
| 9.00 p.m. - 12.00 a.m. | 28 | 65 | 0 |

*Note.* The temperature and relative humidity were derived from the statistic data of weather in Agronomy field, Faculty of Agriculture, Khon Kaen University, during March - May 2016 and 2017
*The distance of light intensity was 5 cm. far from the light source incident upper leaf

Table 4
*The combined treatment of temperature and light intensity in controlled temperature chamber (VRV. Corp., Ltd, Thailand) (T41L1800) was set up at 41°C during 12.00 p.m. - 3.00 p.m. and at 1800 µmol $m^{-2}s^{-1}$ during 10.00 a.m. – 3.00 p.m., respectively and relative humidity was in the ranged between 40 – 80% during 12.00 a.m. – 12.00 p.m.*

| Diurnal time (hr) | Temperature (°C) | Relative humidity (%) | Light intensity* (µmol $m^{-2}s^{-1}$) |
|---|---|---|---|
| 12.00 a.m. - 3.00 a.m. | 28 | 65 | 0 |
| 3.00 a.m. - 7.00 a.m. | 25 | 80 | 0 |
| 7.00 a.m. - 9.00 a.m. | 30 | 65 | 700 |
| 9.00 a.m. - 10.00 a.m. | 35 | 56 | 700 |
| 10.00 a.m. - 12.00 p.m. | 38 | 45 | 1,800 |
| 12.00 p.m. - 3.00 p.m. | 41 | 40 | 1,800 |
| 3.00 p.m. - 5.00 p.m. | 38 | 45 | 700 |
| 5.00 p.m. - 6.00 p.m. | 35 | 50 | 700 |
| 6.00 p.m. - 9.00 p.m. | 32 | 55 | 0 |
| 9.00 p.m. - 12.00 a.m. | 28 | 65 | 0 |

*Note*. The temperature and relative humidity were derived from the statistic data of weather in Agronomy field, Faculty of Agriculture, Khon Kaen University, during March - May 2016 and 2017
*The distance of light intensity was 5 cm. far from the light source incident upper leaf

**Determination of Leaf Gas Exchange and PSII Efficiency**

After *D. alatus* seedlings treated with combined conditions for 7 days, these seedlings were determined with leaf gas exchange and PSII efficiency. Parameters of leaf gas exchange (net photosynthetic rate; *A*, stomatal conductance; $g_s$, transpiration rate; *E*, intercellular $CO_2$ concentration; $C_i$ and vapor pressure deficit; *Vpd*) in *D. alatus* seedlings were measured by using Licor–6400XT Portable Photosynthesis System (LICOR Inc., Lincoln, Nebraska, USA). All leaf gas exchange parameters were investigated in young fully expanded leaf at a middle part of leaf. The conditions of leaf gas exchange measurement were set up as following: leaf temperature at 30°C, relative humidity at 70%, ambient atmospheric $CO_2$ concentration at 400 ppm and light intensity at 700 µmol $m^{-2}s^{-1}$. For investigation of $A/C_i$ curve was set up $CO_2$ concentrations as 0, 50, 100, 200, 400, 500, 800 and 1,000 ppm under light intensity at 700 µmol $m^{-2}s^{-1}$.

Parameters of PSII efficiency measurement (minimal fluorescence in light adapted state; $F_o'$, maximal fluorescence in light adapted state; $F_m'$, Steady state fluorescence; $F_s$, effective quantum yield of PSII efficiency; $\Delta F/F_m'$, variable to maximum quantum yield of PSII; $F_v'/F_m'$ and electron transport rate; ETR) were carried out by using Licor–6400XT. PSII efficiency parameters were calculated according to Schreiber (2004). Portable Photosynthesis System (LICOR Inc., Lincoln, Nebraska, USA). All their measurements were investigated concomitant with the same leaf gas exchange measurement.

**Determination of Photosynthetic Pigment Contents**

Photosynthetic pigment contents (total chlorophyll; Chl *a*+*b*, chlorophyll a; Chl *a*, chlorophyll b; Chl *b* and carotenoid; *Car*) were determined according to Arnon (1949). Leaf samples (0.1 g) were cut into small pieces and put in a glass test tube and soaked in 10 ml of 80% acetone for 72 hr in darkness. The solution was filtered with Whatman filter paper No.1 and then total extraction volume (V) was recorded. The absorbance was measured at OD440, OD645 and OD663 by using a UV-Vis spectrophotometer (Model i3, Jinan Hanon Instruments co., Ltd, China) and using 80% acetone as a blank. All photosynthetic pigment parameters were calculated using the equations below as described by Arnon (1949) and were expressed in mg/g fresh weight (mg/gFW). The carotenoid (*Car*) content was calculated using the following Bajracharya (1999):

$$\text{Chl } a+b = [20.2(OD645) + 8.02(OD663)] \times V/(1000 \times W)$$

$$\text{Chl } a = [12.7(OD663) - 2.69(OD645)] \times V/(1000 \times W)$$

$$\text{Chl } b = [22.9(OD645) - 4.68(OD663)] \times V/(1000 \times W)$$

$$Car = (4.69 \times OD440) - [0.268(20.2 \times OD645) + (8.02 \times OD663)] \times V/(1000 \times W)$$

### Statistical Data Analysis

The experiment was designed in completely randomized design (CRD) with 5 replications. The significant difference data between different treatments were analyzed by one way analysis of variance (ANOVA) and Duncan's new multiple range test (DMRT) at $p \leq 0.05$ by using SPSS Window version 16.0.

## RESULTS AND DISCUSSIONS

### Effect of High Temperature Stress and Light Stress on Morphology of Young *Dipterocarpus alatus*

The effect of temperature and light intensity on morphology of young *D. alatus* treated with temperature at 35 or 41°C and light intensity at 700 or 1800 µmol $m^{-2}s^{-1}$ was shown in Figure 1 and Table 5. Leaf characteristic of young *D. alatus* after treatment with temperature and light intensity resulted in mild heat injury indicated by a small burn and necrosis at leaf tip in T35L1800 and severe heat injury indicated by a large burn and necrosis at middle and base of leaf in T41L1800 (Figure 1). However, in both of T35L700 and T41L700 leaf morphology was unaffected after treatment under these conditions. Therefore, these results suggested that high light intensity (1800 µmol $m^{-2}s^{-1}$) induced leaf injury indicated by burning and necrosis lesions. The combination stress between high temperature (41°C) and high light intensity (1800 µmol $m^{-2}s^{-1}$) induced enlarged leaf injury compared to temperature at 35°C and high light intensity at 1800 µmol $m^{-2}s^{-1}$. The number of leaves and height showed no significant difference in all treatments. However, leaf numbers and height were decreased with increasing temperature and light intensity as shown in Table 5. The highest leaf numbers and height were found in treatment T35L700 approximately 5.96±1.19 leaves and 2.21±1.35 cm, respectively. Light intensity is an important requirement factor for plant growth and development. In addition, different plant species require

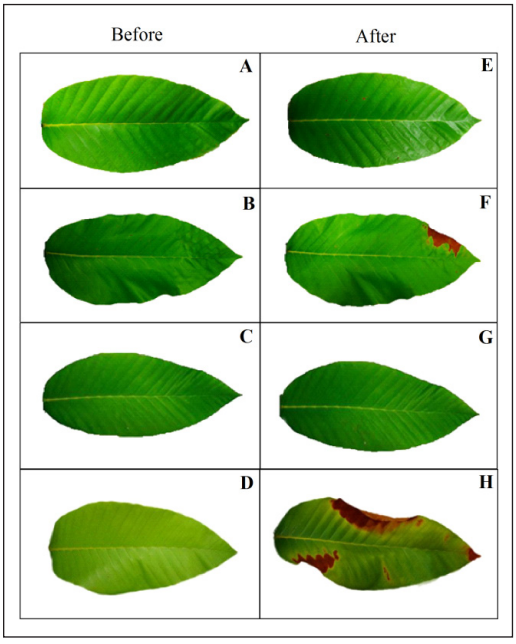


*Figure 1. Dipterocarpus alatus* leaves showed heat injury symptoms after treatment with different temperatures and different light intensity. A-D showed *D. alantus* leaf before being treated with combined stress and E-H showed *D. alantus* leaf after treated with combined stress at 7 days. A and E indicated *D. alantus* leaf treated with temperature at 35°C and light intensity at 700 µmol $m^{-2}s^{-1}$. B and F indicated *D. alantus* leaf treated with temperature at 35°C and light intensity at 1800 µmol $m^{-2}s^{-1}$. C and G indicated *D. alantus* leaf treated with temperature at 41°C and light intensity at 700 µmol $m^{-2}s^{-1}$. D and H indicated *D. alantus* leaf treated with temperature at 41°C and light intensity at 1800 µmol $m^{-2}s^{-1}$

Table 5
*Reduction percentage of number of leaves, height, leaf angle, leaf area and % heat injury after treated with combined stress. The values were means ± SE; n = 3 - 4*

| Treatment | | | % Reduction | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | Temperature (°C) | Light intensity (µmol $m^{-2}s^{-1}$) | Number of leaves | Height (cm) | Leaf area ($cm^2$) | Leaf angle | % Heat injury |
| T35L700 | 35 | 700 | +5.96±1.19 | +2.21±1.35 | +1.33±0.07$^a$ | +3.83±0.49$^a$ | +0.07±0.00$^a$ |
| T35L1800 | 35 | 1800 | +4.44±4.44 | +1.44±0.53 | -0.84±0.34$^b$ | +1.21±1. 59$^a$ | +0.46±0.18$^a$ |
| T41L700 | 41 | 700 | +2.22±2.22 | +0.62±0.36 | +0.78±0.35$^a$ | +1.43±0.79$^a$ | +0.21±0.04$^a$ |
| T41L1800 | 41 | 1800 | +1.08±1.08 | +0.80±0.40 | -1.04±0.38$^b$ | -8.77±2.82$^b$ | +3.01±0.81$^b$ |
| | Mean | | 3.43 | 1.27 | 0.06 | -0.57 | 0.94 |
| | F-test | | ns | ns | ** | ** | ** |

*Note*. ns indicated no significance and the different small letters in the same column indicated significant difference between treatments by Duncan multiple range test (DMRT) at $p \leq 0.05$

various light intensity because light intensity can directly affect physiological and morphological plant adaptation (Böhnke & Bruelheide, 2013). Percentages of reduction in leaf area, leaf angle and heat injury were found significantly different in all treatments. The values of leaf area and leaf angle were decreased with increasing in temperature and light intensity (at 41°C and 1800 µmol $m^{-2}s^{-1}$; T41L1800) which were found approximately -8.77±2.82% and -1.04±0.38%, respectively. However, percentage of heat injury was found increased with increasing in temperature and light intensity (Table 5). The highest percentage of heat injury was found at the highest temperature at 41°C and the highest light intensity at 1800 µmol $m^{-2}s^{-1}$ (Table 5). This suggested that high temperature and high light intensity degraded and damaged chlorophyll molecules resulted in cell damage. These caused leaf necrosis symptoms that we called heat injury as shown in Figure 1H.

## Effect of High Temperature Stress and Light Stress on Gas Exchange of Young *Dipterocarpus alatus*

The performance of photosynthesis in gas exchange of young *D. alatus* is shown in Figure 2. Net photosynthetic rate (*A*) in young *D. alatus* leaf treated with temperature at 35°C and light intensity at 1800 µmol $m^{-2}s^{-1}$ (T35L1800) and temperature at 41°C and light intensity at 1800 µmol $m^{-2}s^{-1}$(T41L1800) exhibited significantly lower than those treated with temperature at 35°C and light intensity at 700 µmol $m^{-2}s^{-1}$ (T35L700) and temperature at 41°C and light intensity at 700 µmol $m^{-2}s^{-1}$ (T41L700), respectively (Figure 2A). The lowest value of *A* was found in T41L1800 (approximately 3.99±0.62 µmol $CO_2$ $m^{-2}s^{-1}$). This suggested that strong irradiation was more influenced in *A* than high temperature. This result is according to some studies showing strong irradiation reduced gas exchange parameters. Ping et al. (2015) reported that apple leaf (*Malus domestica*

Borkh.) exhibited a marked decline in net photosynthetic rate (*A*) and Rubisco activity under strong irradiation at 100%. However, this study showed that the combination stress between high temperature and strong irradiation stimulated a marked decrease in *A* indicated by the lowest value of *A* in T41L1800. For stomatal conductance value ($g_s$), the combination stress between high temperature at 41°C and strong irradiation at 1800 µmol $m^{-2}s^{-1}$ (T41L1800) induced a significant decrease in $g_s$ (0.04±0.01 mol $H_2O$ $m^{-2}s^{-1}$) compared to other treatment such as T35L1800 (0.10±0.02 mol $H_2O$ $m^{-2}s^{-1}$) and T41L700 (0.10±0.01 mol $H_2O$ $m^{-2}s^{-1}$) (Figure 2B). This result suggested that strong combination stress between high temperature and strong irradiation influenced stomata closure in young *D. alatus*. Other parameters such as transpiration rate (*E*), intercellular $CO_2$ concentration ($C_i$) and vapor pressure deficit (*Vpd*) found no significant difference between treatments as shown in Figures 2C, 2D and 2E, respectively. For trend of

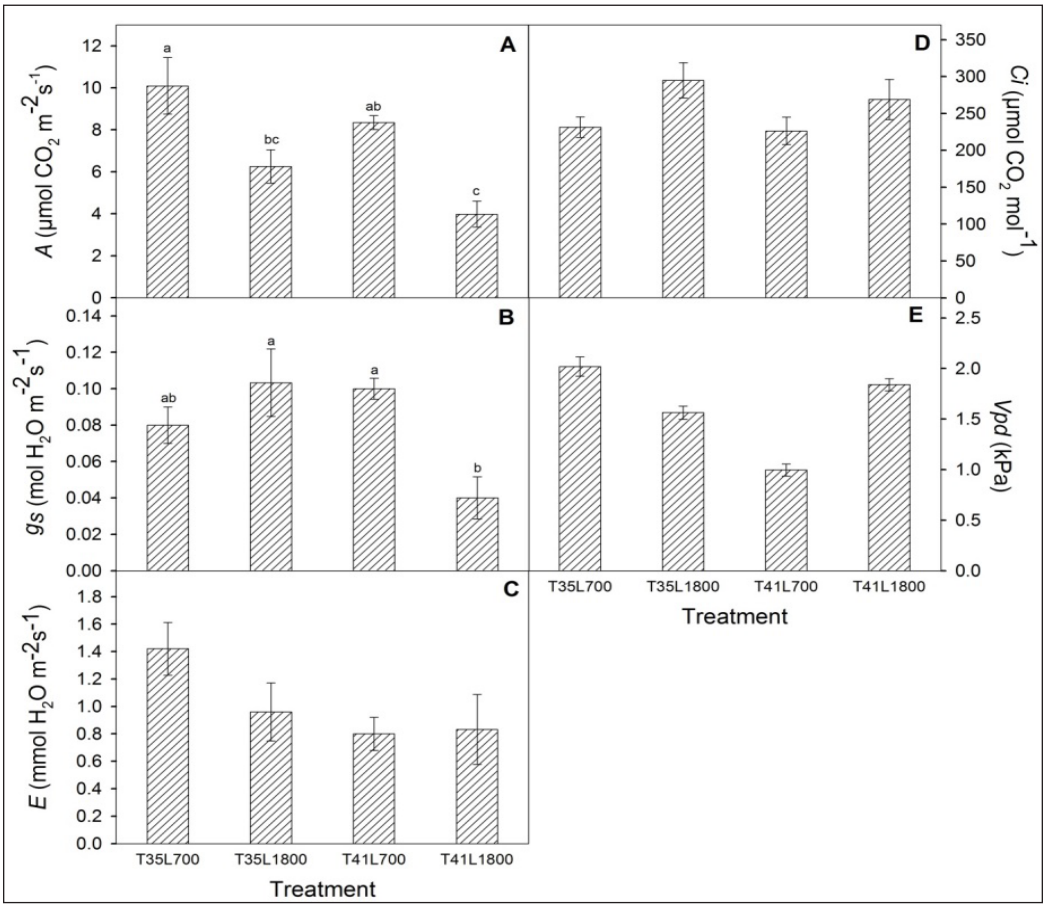


*Figure 2*. Effect of temperature (35°C; T35 or 41°C; T41) and irradiation (700 µmol $m^{-2}s^{-1}$; L700 or 1800 µmol $m^{-2}s^{-1}$; L1800) on net photosynthetic rate; *A* (A), stomatal conductance; $g_s$ (B), transpiration rate; *E* (C), intercellular $CO_2$ concentration; $C_i$ (D) and vapor pressure deficit; *Vpd* (E) in young *Dipterocarpus alatus* leaf after 7 days. The values were mean ± SE; n = 3 - 4

*E* value showed decreased with increased in temperature and irradiation (Figure 2C). The intercellular $CO_2$ concentration was higher in strong irradiation combined with normal- and high temperature (Figure 2D). For *Vpd* values exhibited fluctuations in between treatments (Figure 2E). This suggested that *Vpd* values depended on air humidity during measurement not influenced from temperature and irradiation treatment. From photosynthetic results, we indicated that high temperature and strong irradiation affected markedly lower rate of photosynthesis and transpiration in young *D. alatus* by stomatal closure, even though there was a high intercellular $CO_2$ concentration.

## Effect of High Temperature Stress and Light Stress on PSII Efficiency of Young *Dipterocarpus alatus*

Photosynthetic efficiency of PSII parameters in young *D. alatus* is shown in Figure 3. The trend of effective quantum yield of PSII efficiency, $\Delta F/F_m'$ and variable to maximum quantum yield of PSII, $F_v'/F_m'$

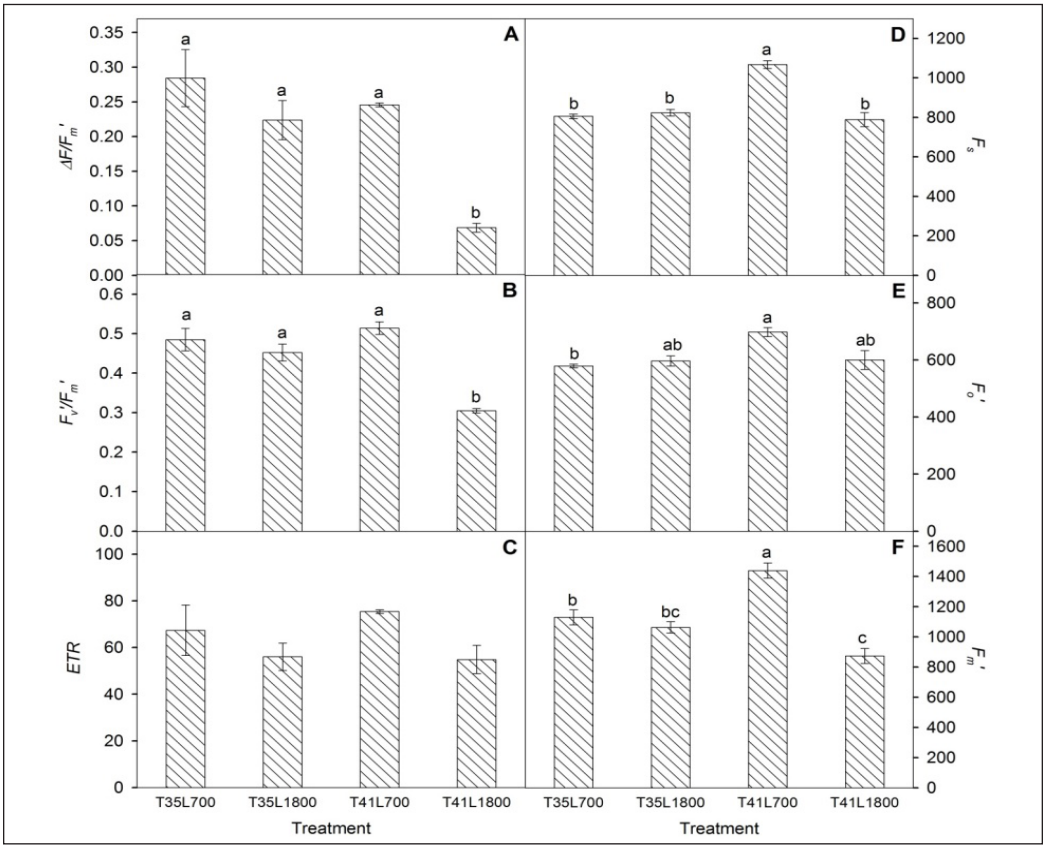


*Figure 3*. Effect of temperature (35°C; T35 or 41°C; T41) and irradiation (700 µmol $m^{-2}s^{-1}$; L700 or 1800 µmol $m^{-2}s^{-1}$; L1800) on effective quantum yield of PSII efficiency; $\Delta F/F_m'$ (A), effective maximum quantum yield of PSII; $F_v'/F_m'$ (B), electron transport rate; *ETR* (C), steady state fluorescence; $F_s$ (D), minimal fluorescence in light adapted state; $F_o'$ (E) and maximal fluorescence in light adapted state; $F_m'$ (F), in young *Dipterocarpus alatus* leaf. The values were mean ± SE; n = 3 - 4

was significantly declined with increased temperature at 41°C and strong irradiation at 1800 µmol $m^{-2}s^{-1}$ (T41L1800) as shown in Figures 3A and B. The slightly reduction of electron transport rate value was found in young *D. alatus* treated with strong irradiation at 1800 µmol $m^{-2}s^{-1}$ combined with temperature at 35 or 41°C (Figure 3C). In addition, the lowest of maximal fluorescence in light adapted state, $F_m'$ was found in young *D. alatus* treated with high temperature at 41°C and strong irradiation at 1800 µmol $m^{-2}s^{-1}$ (T41L1800) as shown in Figure 3F. The higher of all PSII efficiency parameters ($\Delta F/F_m'$, $F_v'/F_m'$, ETR, $F_s$, $F_o'$ and $F_m'$) was found in T41L700 compared to other treatments. These results suggested that high temperature combined with strong irradiation affected photosynthetic light reaction by reduced mechanism of PSII efficiency and electron transport. However, high temperature combined with normal irradiation did not affect photosynthetic efficiency of PSII. As a result, combined stress between high temperature and strong irradiation influenced reduced photosynthetic mechanism at PSII more than individual stress.

### Effect of High Temperature Stress and Light Stress on Photosynthetic Pigment of Young *Dipterocarpus alatus*

The effect of temperature and light intensity on photosynthetic pigment contents in young *D. alatus* is shown in Table 6. Photosynthetic pigment contents were investigated in terms of total chlorophyll (Chl *a*+*b*), chlorophyll a (Chl *a*), chlorophyll b (Chl *b*) and carotenoid (*Car*) after 7 days treated with temperature at 35 or 41°C and irradiation at 700 or 1800 µmol $m^{-2}s^{-1}$. The contents of Chl *a*+*b* and Chl *a* were found markedly reduced with increased temperature combined with strong irradiation (T41L1800) compared to other treatments (Table 6). For Chl *b* and *Car* treated with high temperature and strong irradiation (T41L1800) exhibited no significant difference compared to other treatments by slightly reduction. These results suggested that the combination stress between high temperature and strong irradiation affected photosynthetic pigments by reducing total chlorophyll and Chl *a* contents in contrast to Chl *b* and *Car* contents which were unaffected from combination stress. Because *Car* worked as an antioxidant for plant protecting it from environmental stress especially high sunlight. Group of *Car* protected plants from high sunlight such as zeaxanthin (Dongsansuk et al., 2013). High temperature affects chloroplast function and decrease in chlorophyll contents (Paethaisong et al., 2019; Pansarakham et al., 2018; Purnama et al., 2018; Xu et al., 1995). These resulted in changing in physiological process (Björkman et al., 1980; Paethaisong et al., 2019) and reduction in plant photosynthetic rate (Berry & Raison, 1981; Dongsansuk et al., 2017) while our results showed combination stress affected photosynthetic function such as photosynthetic rate and PSII efficiency. Phonguodume et al. (2012) showed 5 tropical trees such as *Anisoptera costata*, *Afzelia xylocarpa*, *Dipterocarpus alatus*, *Dalbergia Cochinchinensis* and

Table 6
*Effect of high temperature and strong irradiation on photosynthetic pigment changing in young* Dipterocarpus alatus *leaves. The values were mean ± SE, n = 3 – 4*

| Treatment | | Photosynthetic pigment contents (mg/g FW) | | | |
|---|---|---|---|---|---|
| Temperature (ºC) | Light intensity (µmol $m^{-2}s^{-1}$) | Chl *a*+*b* | Chl *a* | Chl *b* | *Car* |
| 35 | 700 | 0.921±0.05 | 0.632±0.04 | 0.248±0.01 | 0.259±0.01 |
| 35 | 1800 | 0.798±0.12 | 0.573±0.09 | 0.212±0.03 | 0.224±0.00 |
| 41 | 700 | 0.709±0.06 | 0.499±0.05 | 0.199±0.01 | 0.229±0.01 |
| 41 | 1800 | 0.602±0.08 | 0.390±0.07 | 0.201±0.02 | 0.250±0.02 |
| Mean | | 0.757 | 0.524 | 0.215 | 0.240 |
| F-test | | ns | ns | ns | ns |

*Note.* ns indicated no significant difference at $p \leq 0.05$ and the different small letters in the same column indicated significant difference between treatments by Duncan multiple range test (DMRT) at $p \leq 0.05$

*Hopea odorata* when exposed to 30-50%, 50-70% and 100% of light intensity had their total chlorophyll contents changed significantly in different species.

## CONCLUSION

The results concluded that morphology and physiology in young *Dipterocarpus alatus* leaf showed severe injury under high temperature at 41°C and strong light intensity at 1800 µmol $m^{-2}s^{-1}$. These were indicated by leaf burned and injured and also reduced significantly different physiological processes such as reduction in photosynthetic rate and photosynthetic pigment contents. Therefore, young *D. alatus* leaf (2-year-old) was sensitive to the combination stress between heat and excessive light.

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# Antipyretic Effect of Mitragynine and Crude Methanolic Extract of *Mitragyna speciosa* Korth. in Mice

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## ABSTRACT

*Mitragyna speciosa* Korth., also known as *ketum* or *kratom*, is a tropical plant native to Southeast Asia. Mitragynine is its major active alkaloid. It is traditionally used as treatment for various conditions, including fever. The crude extract of *M. speciosa* leaves has been proven to have anti-inflammatory and analgesic properties. In general, *M. speciosa* induces a dose-dependent effect, inducing a stimulant effect at low dose and an opioid-like effect at a high dose. This study was conducted to determine the antipyretic effect of mitragynine and methanolic extract of *M. speciosa* (MSM) using mice as an *in vivo* pyretic model. Eighty mice were divided into 8 groups: 6 treatment groups (mitragynine: 5, 10, and 20 mg/kg; MSM: 50, 100, and 200 mg/kg) and 2 control groups (20% Tween 80 in 0.9% NaCl; ketoprofen 1 mg/kg). Eighteen hours after induction of pyrexia by inoculation of yeast, rectal temperature was measured every half an hour for 5 hours. Compared to the negative control group, all groups treated with either mitragynine or MSM had significant reduction of rectal temperature at different points of time. The positive control group treated with ketoprofen had significant ($P < 0.001$) reduction of pyrexia from 0.5 to 5.0 hours after dosing. At 200 mg/kg, MSM has led to the opioid-like effect of hypothermia, possibly due to its synergistic effect with other compounds such as 7-hydroxymitragynine or mitragynine pseudoindoxyl. This article discusses concerns pertaining to toxicity of mitragynine and MSM, and possible involvement of cyclooxygenase and microsomal prostaglandin E2 synthase pathways. In conclusion, mitragynine and MSM possess dose-dependent antipyretic properties in mice.

*Keywords*: *Mitragyna speciosa*, mitragynine, mice, pyrexia

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## INTRODUCTION

*Mitragyna speciosa* Korth., also known as *ketum* or *kratom*, is a plant found in tropical and subtropical Asia (such as Thailand, Indonesia, and Malaysia), East and West Africa, and India (Ahmad & Aziz, 2012). In Malaysia, it is widely distributed across the northern half of Peninsular Malaysia and Selangor (Idayu et al., 2011). More than 40 different alkaloids have been identified in the plant (Meireles et al., 2019). Mitragynine is regarded as the major alkaloid of the whole leaf, amounting to about 66.2% (Gibbons & Arunotayanun, 2013).

Asian workers use the leaves of *M. speciosa* for their stimulant effects (Saingam et al., 2013). The stimulant effect enhances the workers' tolerance to endure heavy work and also relieves muscle strains. In Thailand and Malaysia, it is also used traditionally as treatment for fever (pyrexia), diarrhea, opium addiction, diabetes, and helminthiasis (Ahmad & Aziz, 2012). The leaves have been used to treat opium withdrawal syndrome and are considered as an alternative to methadone or other opioids (Prozialeck, 2016). Administration of *M. speciosa* usually results in dose-dependent effects. An opioid-like effect predominates at high doses, whereas administration at lower doses typically results in stimulant-like effects.

Fever, or pyrexia, is an increase in body temperature above the normal range triggered by the presence of pyrogens. It can be caused by many infectious (e.g., bacterial, viral, parasitic diseases) or noninfectious (e.g., trauma, thrombosis, cancer) medical conditions. Hence, fever is the most common primary complaint in medical practices (Celebi et al., 2009). For humans, most antipyretic drugs are available as nonprescription drugs, including aspirin, acetaminophen, ibuprofen, ketoprofen, and naproxen. Although these antipyretic drugs are considered safe, adverse side effects have been reported that affect the gastrointestinal tract, kidney, and liver (Plaisance, 2000).

Scientifically, it has been shown that mitragynine and the crude methanolic extract of *M. speciosa* (MSM) provide anti-inflammatory and antinociceptive properties by affecting the supraspinal opioid receptors (Meireles et al., 2019; Mossadeq et al., 2009). Most analgesic and anti-inflammatory drugs are known to possess antipyretic activity (Srinivasan et al., 2003). Since *M. speciosa* has been used traditionally as a treatment for fever, it is possible that it possesses an antipyretic effect. However, there is no scientific report available pertaining to such an effect of the crude extract of *M. speciosa* and mitragynine. Such information could be important for human or veterinary therapeutic uses in the future. This study was conducted to validate the traditional claim of the antipyretic effect of MSM and mitragynine using an *in vivo* pyretic mice model. Subsequently, the effective doses, onset of action, and duration of action related to the antipyretic effect were determined.

## MATERIALS AND METHODS

### Preparation of Mitragynine and MSM

The study was conducted in 2010. MSM was obtained from freeze-dried stock of a previous study (Mossadeq et al., 2009). The pure compound of mitragynine (Chromadex Inc., CA, USA) was purchased from Chemtron Biotechnology Sdn. Bhd. (Kuala Lumpur, Malaysia). Each substance was prepared in 20% Tween 80 in 0.9% NaCl (T-80-NaCl) (Idayu et al., 2011). This was achieved by dissolving them in 20 mL Tween 80 using a sonicator. The resulting solution was then mixed with 80 mL of 0.9% NaCl and stirred using a magnetic stirrer to produce a homogenous solution.

### Animals

A total of 80 female BALB/c mice weighing between 20 and 25 g were obtained from the Animal Resource Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The animals were acclimatized for 2 weeks and kept in a standard cage at a stocking density of 8 mice per cage. They were housed in a laboratory animal facility with a 12-h light-dark cycle. The mice were brought to the laboratory and were acclimatized to the laboratory environment for 48 hours (h) prior to the experiment. All experimental procedures were approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universiti Putra Malaysia (FPV/AUP/FYP/009/2010).

### Experimental Design and Antipyretic Effect

Evaluation of the antipyretic effect was conducted as previously described (Zakaria et al., 2008), with minor modifications. The basal temperature of all mice was measured using a digital thermometer by inserting a thermistor probe 1 cm into the rectum. Subsequently, the mice were induced into pyrexia by subcutaneous inoculation of 30% (w/v) suspension of Brewer's yeast in 0.9% NaCl at a dose of 10 mL/kg. Eighteen h after induction of pyrexia, rectal temperature was recorded. Because all 80 mice had more than 0.5°C rise in rectal temperature, they were all subjected to the experiments. They were randomly divided into 8 groups. Groups 1, 2, and 3 were administered 5, 10, or 20 mg/kg mitragynine intraperitoneally (i.p.), respectively. Groups 4, 5, and 6 were administered 50, 100, and 200 MSM i.p., respectively. The different doses of mitragynine and MSM were based on a previous study that evaluated the antinociceptive properties of these substances (Sabetghadam et al., 2010). Group 7 was administered ketoprofen 1 mg/kg i.p. as positive control, and mice of Group 8 were administered T-80-NaCl i.p. as a negative control. Rectal temperature was immediately measured after the treatment and every 30 minutes thereafter, for a total period of 5 h. Percentage of temperature reduction was calculated using the following formula:

$$\text{Percentage of temperature reduction} = \frac{(\text{yeast} - \text{induced pyrexia} - \text{post treatment temperature})}{\text{yeast} - \text{induced pyrexia}} \times 100$$

**Statistical Analysis**

The mean change in the rectal temperature for each mouse was calculated. General linear model repeated measure analysis of variance (ANOVA) was used to analyze data separately between the groups treated with mitragynine and the groups treated with MSM. Results were expressed as mean ± standard error of the mean (SEM). One-way ANOVA followed by posthoc analysis was used to compare data of the treatment and negative control groups at each point of time. All data were analyzed to observe different degrees of statistical significance ($P < 0.05$, $P < 0.01$, $P < 0.001$).

## RESULTS

Mauchly's test of sphericity, multivariate test, and test within subject from repeated measure ANOVA showed a significant ($P < 0.001$) relationship between change in rectal temperature, time, treatment, and interaction between time and treatment. In between subjects, the rectal temperature was found to be significantly affected by the different types of treatment administered, while within subjects, the rectal temperature was significantly affected by the time.

Induction of pyrexia in negative control mice of Group 8 resulted in elevation of rectal temperature to 38°C. This pyretic state persisted throughout the 5 h duration, with the highest rectal temperature of 38.67°C recorded at 1.0 h. In general, mice of all treatment groups showed significant reduction of rectal temperature compared to negative control mice of Group 8 at different points of time (Figures 1 and 2). Positive control mice of Group 7 showed significant ($P < 0.001$) difference in rectal temperature from 0.5 to 5.0 h compared to negative control mice of Group 8 (Figure 1). The same group had a significant reduction in rectal temperature between 0.5 and 1.0 h, and between 3.5 and 5.0 h compared to Group 8 (Tables 1 and 2). The lowest rectal temperature achieved in the positive control mice of Group 7 treated with ketoprofen was 36.65°C at 5.0 h.

Mitragynine administered at a dose of 5 mg/kg in Group 1 mice resulted in significant reduction in rectal temperature between 1.0 and 4.5 h. On the other hand, mice treated with mitragynine (10 mg/kg) in Group 2 showed significant reduction in rectal temperature between 1.0 and 5.0 h. Administration of mitragynine at a dose of 20 mg/kg in mice of Group 3 led to a highly significant ($P < 0.001$) reduction of rectal temperature between 0.5 and 5.0 h.

Mice of Group 4 administered with MSM at a dose of 50 mg/kg showed statistically significant ($P < 0.05$, $P < 0.01$, $P < 0.001$) reductions of rectal temperature between 2.5 and 5.0 h. Mice of Group 5 treated with MSM at a dose of 100 mg/kg had a significant ($P < 0.05$, $P < 0.01$, $P < 0.001$) reduction in rectal temperature at 0.5 h and between 2.0 and 5.0 h. Mice of Group 6 treated with MSM at a dose of 200 mg/kg had a significant ($P < 0.001$) reduction in rectal temperature between 0.5 and 5.0 h.

The administration of ketoprofen in mice of Group 7 resulted in the highest percentage of inhibition of pyrexia (Figures 1 and 2) at 5.0 h. Among all the doses of mitragynine

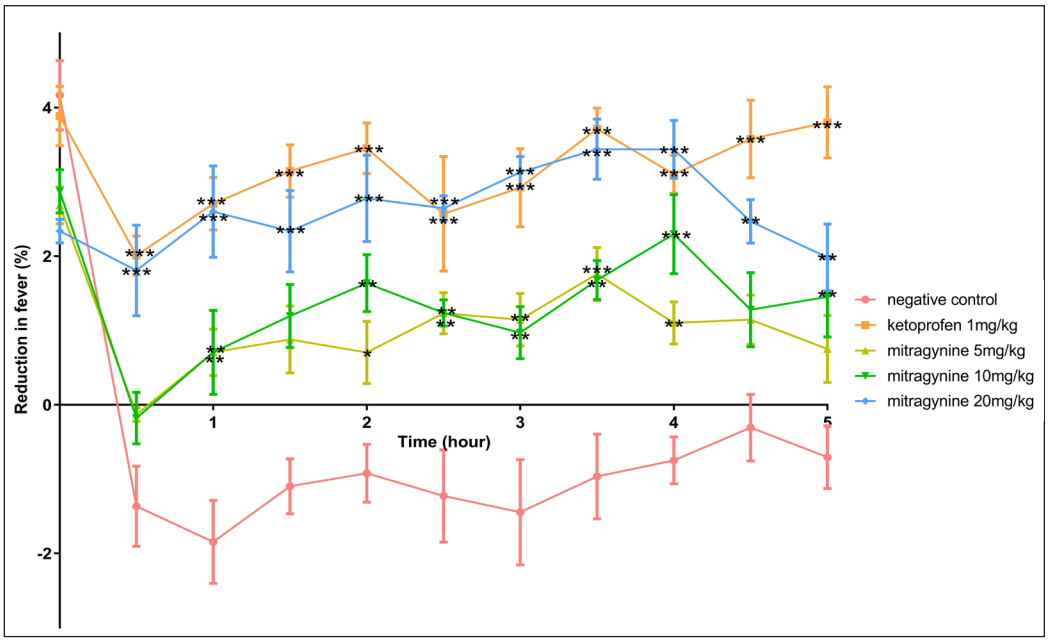


*Figure 1*. Percentage reduction of fever with mitragynine administered intraperitoneally in Brewer's yeast-induced pyrexia in mice. (*P < 0.05, **P < 0.01, and ***P < 0.001 compared to the negative control)

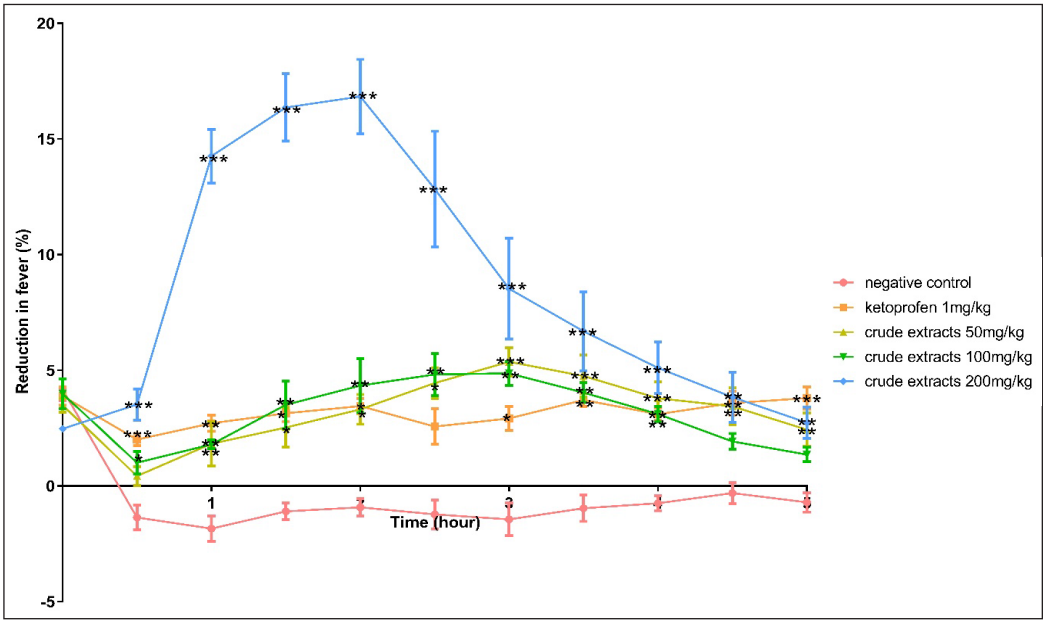


*Figure 2*. Percentage reduction of fever with methanolic extract of *Mitragyna speciosa* (MSM) administered intraperitoneally in Brewer's yeast-induced pyrexia in mice. (*P < 0.05, **P < 0.01, and ***P < 0.001 compared to the negative control)

Table 1

*Mean ± standard error of the mean (SEM) of rectal temperature with mitragynine administered intraperitoneally in Brewer's yeast-induced pyrexia in mice (*P < 0.05, **P < 0.01, and ***P < 0.001 compared to the negative control)*

| Treatments | Before yeast | 18 h after yeast | Time after treatment (hour) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 | 4.0 | 4.5 | 5.0 |
| Negative control | 36.38 ± 0.145 | 37.97 ± 0.109 | 38.48 ± 0.164 | 38.67 ± 0.199 | 38.38 ± 0.168 | 38.32 ± 0.176 | 38.43 ± 0.276 | 38.52 ± 0.313 | 38.33 ± 0.235 | 38.25 ± 0.118 | 38.08 ± 0.199 | 38.23 ± 0.141 |
| Ketoprofen 1 mg/kg | 36.62 ± 0.048 | 38.10 ± 0.132 | 37.33 ± 0.092*** | 37.07 ± 0.067*** | 36.90 ± 0.115*** | 36.78 ± 0.111*** | 37.12 ± 0.189*** | 36.98 ± 0.091*** | 36.68 ± 0.079*** | 36.92 ± 0.060*** | 36.73 ± 0.112*** | 36.65 ± 0.163*** |
| Mitragynine 5 mg/kg | 36.83 ± 0.095 | 37.85 ± 0.067 | 37.90 ± 0.052 | 37.58 ± 0.154*** | 37.52 ± 0.158* | 37.58 ± 0.154* | 37.38 ± 0.114** | 37.42 ± 0.149** | 37.18 ± 0.119*** | 37.43 ± 0.126** | 37.42 ± 0.149* | 37.57 ± 0.228 |
| Mitragynine 10 mg/kg | 36.65 ± 0.165 | 37.73 ± 0.092 | 37.80 ± 0.113* | 37.47 ± 0.225** | 37.28 ± 0.221* | 37.12 ± 0.199*** | 37.27 ± 0.117*** | 37.37 ± 0.099** | 37.10 ± 0.121*** | 36.87 ± 0.220*** | 37.25 ± 0.188** | 37.18 ± 0.149** |
| Mitragynine 20 mg/kg | 36.92 ± 0.075 | 37.80 ± 0.026 | 37.12 ± 0.224*** | 36.82 ± 0.226*** | 36.92 ± 0.204*** | 36.75 ± 0.208*** | 36.80 ± 0.058*** | 36.62 ± 0.080*** | 36.50 ± 0.163*** | 36.50 ± 0.134*** | 36.87 ± 0.095*** | 37.05 ± 0.152*** |

Table 2

*Mean ± standard error of the mean (SEM) of rectal temperature with methanolic extract of* Mitragyna speciosa *(MSM) administered intraperitoneally in Brewer's yeast-induced pyrexia in mice. (*P < 0.05, **P < 0.01, and ***P < 0.001 compared to the negative control)*

| Treatments | Before yeast | 18 h after yeast | Time after treatment (hour) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 | 4.0 | 4.5 | 5.0 |
| Negative control | 36.38 ± 0.145 | 37.97 ± 0.109 | 38.48 ± 0.164 | 38.67 ± 0.199 | 38.38 ± 0.168 | 38.32 ± 0.176 | 38.43 ± 0.276 | 38.52 ± 0.313 | 38.33 ± 0.235 | 38.25 ± 0.118 | 38.08 ± 0.199 | 38.23 ± 0.141 |
| Ketoprofen 1 mg/kg | 36.62 ± 0.048 | 38.10 ± 0.132 | 37.33 ± 0.092*** | 37.07 ± 0.067* | 36.90 ± 0.115 | 36.78 ± 0.111 | 37.12 ± 0.189 | 36.98 ± 0.091 | 36.68 ± 0.079* | 36.92 ± 0.060** | 36.73 ± 0.112** | 36.65 ± 0.163*** |
| MSM 50 mg/kg | 36.82 ± 0.054 | 38.15 ± 0.159 | 37.98 ± 0.105 | 37.45 ± 0.281 | 37.18 ± 0.280 | 36.88 ± 0.220 | 36.45 ± 0.219* | 36.10 ± 0.207** | 36.33 ± 0.304** | 36.70 ± 0.256** | 36.83 ± 0.260** | 37.22 ± 0.180** |
| MSM 100 mg/kg | 36.48 ± 0.172 | 38.00 ± 0.103 | 37.62 ± 0.135** | 37.32 ± 0.070 | 36.67 ± 0.384 | 36.35 ± 0.447* | 36.17 ± 0.340* | 36.15 ± 0.198** | 36.47 ± 0.169** | 36.82 ± 0.095** | 37.27 ± 0.056 | 37.48 ± 0.075* |
| MSM 200 mg/kg | 37.06 ± 0.081 | 38.00 ± 0.055 | 36.66 ± 0.238*** | 32.58 ± 0.418*** | 31.78 ± 0.530*** | 31.60 ± 0.585*** | 33.12 ± 0.936*** | 34.76 ± 0.829*** | 35.46 ± 0.661*** | 36.06 ± 0.432*** | 36.54 ± 0.421** | 36.96 ± 0.268** |

administered in this experiment, the dose of 20 mg/kg in mice of Group 3 showed the highest percentage of temperature reduction, which occurred at 4.0 h (Table 1). As for the groups treated with MSM i.p., the greatest percentage of reduction of rectal temperature was observed in mice of Group 6 at 2.0 h. However, mice of this group showed hypothermia between 1.0 and 3.5 h. In general, both mitragynine and MSM administered intraperitoneally produced a dose-dependent antipyretic effect.

## DISCUSSION

In this study, both mitragynine and MSM administered i.p. were observed to possess antipyretic properties in mice. The results suggested that administration of mitragynine at a dose of 20 mg/kg is marginally a more effective antipyretic compared to ketoprofen. On the other hand, administration of MSM at 200 mg/kg resulted in significantly more antipyretic effect compared to ketoprofen, to the point of adverse effect to the host. This is further discussed in a later paragraph.

Based on the pattern of rectal temperatures throughout the 5 h study, it was observed that the onset of action of both mitragynine and MSM was rapid, approximately 0.5 to 1.0 h after administration. However, in terms of duration of action, both mitragynine and MSM only provided a short duration of antipyretic activity, approximately 3.5 to 4.0 h. Ketoprofen provided a similarly rapid onset of action but with a longer duration of actions (Cossellu et al., 2019) compared to MSM and mitragynine.

Mitragynine and MSM were observed to possess dose-dependent antipyretic effects, where administration at high dose of either mitragynine or MSM resulted in a statistically significant degree of pyrexia inhibition over time. Based on the statistical analysis, and the fact that the temperature decreased from a pyretic state to normal, the effective antipyretic dose for mitragynine was found to be 20 mg/kg, and for MSM it was 100 mg/kg.

At the highest dose of 200 mg/kg tested in this study, MSM resulted in transient hypothermia of the mice, which lasted for 2.5 h. This dose-dependent effect may be explained by the fact that MSM has opioid-like effects at high doses (Babu et al., 2008). Hypothermia is a well-documented effect of opioids (Henderson-Redmond et al., 2016). This may be explained by a synergistic effect brought about by the presence of other compounds in MSM. This leads to a great reduction in body temperature, which subsequently leads to hypothermia. The synergistic effect could be more prominent compared to that of a single compound. A similar synergistic effect was previously postulated in assessment of analgesic properties of MSM (Reanmongkol et al., 2007). It is also possible that hypothermia was due to the presence of another important alkaloid that acts on the opioid receptor, such as 7-hydroxymitragynine or mitragynine pseudoindoxyl. The 7-hydroxymitragynine possesses higher affinity and is 1000 times more potent than morphine, particularly to the μ-opioid receptors, despite there being less of it compared to mitragynine

(McCurdy & Scully, 2005). On the other hand, mitragynine pseudoindoxyl was previously found to be 35-fold more potent than morphine in inhibiting electrically stimulated ileum contraction (Adkins et al., 2011). This potent alkaloid may act agonistically at the μ-opioid receptors, which are present abundantly especially in the hypothalamus, to affect the thermoregulation system of the body. This can produce antipyretic effects at the first 30 minutes post treatment, and then induce hypothermia.

Hypothermia after i.p. administration of 200 mg/kg MSM is an undesirable antipyretic effect related to MSM. Another undesirable effect that could be of concern is toxicity. Previously, a subchronic study concluded that mitragynine is relatively safe at doses of 1-10 mg/kg, but leads to a toxic effect at 100 mg/kg (Sabetghadam et al., 2013). In an acute toxicity study, rodents orally administered 1000 mg/kg of MSM exhibited a reversible effect, with hepato- and nephron-toxicity (Harizal et al., 2010). This is in agreement with the few reports pertaining to *M. speciosa* intoxication in humans (Domingo et al., 2017). In other reports, the role of mitragynine in human fatalities was not conclusive (Domingo et al., 2017; Holler et al., 2011). Although MSM and mitragynine have therapeutic potential, issues pertaining to toxicity and abuse of these substances require further clarification.

Yeast-induced hyperthermia is also known as "fever" or "pyrexia". Its pathogenesis includes the presence of exogenous pyrogens. These will eventually evoke the host-activated macrophages and mast cells to produce pyrogenic cytokines that act on the hypothalamus to induce a fever response (Werner et al., 2006). These cytokines will trigger another important mediator of fever, $PGE_2$ in the thermoregulatory center at the region of the preoptic nuclei of the anterior hypothalamus (Roth & Souza, 2001). $PGE_2$ activates the endocrine, autonomic, and behavioral responses that lead to fever (Saper & Breder, 1994). $PGE_2$ requires the action of cyclooxygenase (COX) and microsomal PGE synthase (mPGES). Ketoprofen is known to inhibit pyrexia partially by inhibition of the COX enzyme. Hence, it is possible that the modes of action of both mitragynine and MSM involve a mechanism similar to that of ketoprofen. However, more studies are needed to ascertain the antipyretic mechanism of action of mitragynine and MSM.

## CONCLUSION

Both mitragynine and MSM possess a dose-dependent antipyretic effect, where i.p. administration of MSM results in an opioid-like effect. Effective doses to achieve a desirable antipyretic effect for mitragynine and MSM are 20 and 100 mg/kg, respectively. The onset of the antipyretic effect of mitragynine and MSM is rapid, but the duration of the antipyretic effect is short.

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## AUTHORS' CONTRIBUTION

Annas Salleh planned and conducted the study, prepared the initial manuscript. Wan Mastura Shaik Mohamed Mossadeq provided the MSM and revised the manuscript. Arifah Kadir planned the study and revised the manuscript.

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# Foraging Activity of the Re-introduced Milky Storks (*Mycteria cinerea*) in Kuala Gula Bird Sanctuary, Perak, Malaysia

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## ABSTRACT

The milky stork is an endangered waterbird species that is currently being re-introduced in the Kuala Gula Bird Sanctuary, Perak, Malaysia. However, little information is available on the re-introduced population's adaptation and activity especially of that related to foraging. To fill this gap, a new group of re-introduced population released between 2013 and 2014 was followed and studied. During the early release period (January – February 2013), the population incorporated the natural sites (intertidal areas, mudflats, and riverbeds) and also the shrimp farms almost equally as their foraging sites (~50% each). Later (March – May 2013), a shift from the natural foraging sites to the shrimp farms could be observed with increasing visits made to the latter area. However, the storks incorporated the natural sites again between June and August 2013 most notably during their breeding activity. Nonetheless, there was a significant reliance on the newly built shrimp farms (monthly mean visits =17.6 ± 1.26, $p = 0.001$) and a high percentage of shrimp consumption (30 - 48%) compared to other prey was recorded in the subsequent period (September - June 2014). Furthermore, the principal component analysis (PCA) indicates that the foraging activity of the waterbirds was more likely tied to the area or size of the foraging sites which were heavily influenced by the anthropogenic activity in Kuala Gula. In addition, there is a concern over the prolonged utilization of the shrimp farms and their resource as the milky storks could be exposed to several hazardous pollutants in the long run.

*Keywords*: Ecology, endangered species, foraging activity, Kuala Gula Bird Sanctuary, milky stork, principal component analysis

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## INTRODUCTION

Waterbirds are often used to monitor environmental variations either temporal or spatial at both species and community levels (Abraham & Sydeman 2004; Rendón-Martos et al., 2000). Their sensitivity to environmental changes can be reflected by their movement or migration activity, making them an excellent bio-indicator of our ecosystem as reviewed by Rahman and Ismail (2018). As such, the availability of suitable habitats for foraging, nesting and roosting are important to the waterbirds' survival. Any disturbances or deterioration in the birds' foraging habitat could cause them to seek a new area for food sources. This would lead to changes in the foragers' diet which often follows the optimal foraging theory. Moreover, the shifting of foraging activity to a new area may occur either temporarily to compensate the low abundance of prey in the original habitat or permanently if its quality further deteriorates. Likewise, the coastal area has been subjected to continuous development throughout the years, causing more problems to the waterbirds population globally due to the alteration of the landscape and their habitats (Ma et al., 2019; Wen et al., 2016).

The Southeast Asia coast, in particular, has been subjected to intense development and urbanization activities (Ramesh et al., 2011; Wong, 1998). The reduction of mudflats and mangroves along its coast is causing many wildlife species to rapidly decline and become endangered. The milky stork, *Mycteria cinerea* (Raffles 1822) is a good example of where the deforestation and development along the coast have driven the population to an endangered status (BirdLife International, 2019; Ismail & Rahman, 2012). The milky stork is a large wading bird from the Ciconiidae family and was once predominantly found along the coast of the Southeast Asia region from Cambodia, southeast Thailand to the west coast of peninsular Malaysia and Indonesia. In Malaysia, the wild population was last observed in 2010 and with the ongoing declining pattern; it is viewed that the wild population may already be extinct (Ismail et al., 2010). The individuals sighted in the country today are most likely to be the ones released in previous re-introduction programs in the Kuala Selangor Nature Park as well as those in the Kuala Gula Bird Sanctuary. The decreasing pattern of the species has also been reported in Indonesia and Cambodia (Iqbal et al., 2012; Naing, 2007). As such, the species has been listed as an endangered species in the IUCN red list (BirdLife International, 2019). Consequently, in 2007 a re-introduction program was carried out in Kuala Gula, Perak, Malaysia to help re-establish the population back into the wild. Nonetheless, little information is available on the milky stork's adaptation and ecology post-release and this could be a hindrance to future conservation efforts for the species and its habitat (Ismail et al., 2012; Ismail & Rahman 2012, 2016). Therefore, this study was conducted to understand the milky stork's foraging activity including the utilization of and preferred foraging areas as well as their diet post-release. There is

hope that the findings will provide important information to support the protection of the milky stork's habitat in the future.

## MATERIALS AND METHODS

### Study Area

The study was conducted in the Kuala Gula Bird Sanctuary in Perak (4°56'00" N; 100°28'00" E) which is part of the larger Matang Mangrove Forest in Malaysia (Figure 1). Elevation of the area averages at 2-3 meters above sea level with average annual rainfall of 3500-4800 mm. It consists of several small fishing villages along the mangroves, covering approximately 10 kilometers of the Kuala Gula coast. Its inland areas have been mostly developed for agricultural activity particularly oil palm plantations. The aquaculture industry is also a booming industry in Kuala Gula. This has led to the increasing number of reclaimed mangroves in recent years (Ismail & Rahman, 2016). Nonetheless, Kuala Gula and its mudflat areas are still regarded as one of the important stopovers in the East Asia-Australia flyway. Between 4,000 and 8,000 individual migratory shorebirds have been recorded to stop and refuel in the area (Lomoljo, 2011). Kuala Gula is also home to important endangered waterbird species such as the wild milky stork (*Mycteria cinerea*) and lesser adjutant (*Leptoptilos javanicus*). Currently, Kuala Gula is the center of the milky stork re-introduction program in Malaysia. According to Ismail and Rahman (2016), Zoo Negara under the management of the Malaysian Zoological Society has agreed to supply a total of 150 captive-bred milky stork individuals to reestablish the population in Kuala Gula. Meanwhile, the Department of Wildlife and Parks (DWNP) has been tasked with monitoring and protecting the re-introduced


*Figure 1*. Map of the study sites in Kuala Gula Bird Sanctuary, Perak, Malaysia

population under existing legislation and acts. The captive-bred individuals will be continuously supplied in small batches, between 5-8 individuals, to the release center, the DWNP office. To date, at least 50 captive-bred individuals have been released in Kuala Gula.

**Sampling Activity**

A newly released group of milky storks obtained from Zoo Negara was followed to its respective foraging sites in the Kuala Gula Bird Sanctuary between January 2013 and December 2014 (2 years). The flight directions of the milky storks from the release center were observed and monitored using the vanishing bearing method (Kenyon, 2006). This method employed a bearing compass to accurately determine the storks' flight directions, assuming that to minimize energy cost, the storks would fly a direct route between its colony (in this case the release center) and their foraging sites. In addition, like other colonial waterbirds, the milky storks are known to congregate while foraging and roosting. Accordingly, the newly released storks congregated with the more experienced individuals from the older groups or batches sent. These individuals can be distinguished by the color and condition of the rings attached to their legs; the new ones are more vivid and unstained compared to individuals from the older groups. No effort was made to distinguish between the age one and two years old individuals as they were observed as one group. In total, eight individuals were observed from the new group. A study done by Ismail et al. (2012) suggested that the species particularly the captive ones were not very active as they spent most of their time roosting (45%) followed by foraging (30%). As such, sampling activity is straightforward as researchers could monitor them without missing any of the released individuals for the above reasons highlighted. A five-day observation between 0700 and 1900 hours was conducted every two weeks for each month between the periods of January 2013 and December 2014. A binocular (Nikon Egret II 8x40CF), a digital SLR camera (80-300mm lenses) and a video recorder (Sony HDR-PJ340) were used during the observations. The number of visits made by the milky storks, distance from the release center, height of trees in foraging areas, disturbances and the prey captured were also recorded. The storks foraging activity could last for several hours hence several recording sessions were taken during each sampling activity with 1-2 hours recorded for each session. No classification or categorization was made for the foraging activity in this study. The preys captured by the storks were identified up to the species level whenever possible and unknown food items taken were classified as 'unidentified' in this study. Observation distances were maintained between 10 and 100 meters. The descriptions of the foraging sites utilized by the storks are shown in Table 1. These sites include both the natural and artificial habitats. The later's hydrology and biotic components have been altered mainly for economic activity. Tree heights were measured using trigonometry method

Table 1
*Coordinates and description of the study sites in Kuala Gula*

| Site | Coordinates (Latitude, Longitude) | Area description |
|---|---|---|
| I | 4° 56' 2.47'' N, 100° 29' 16.41'' E | Newly developed shrimp farms surrounded by mangrove forest |
| II | 4° 56' 26.38'' N, 100° 28' 7.60'' E | A small strip of mangrove forest with heavy anthropogenic activity i.e. boating |
| III | 4° 57' 18.13'' N, 100° 29' 19.37'' E | Mangrove forest turned into shrimp farm |
| IV | 4° 55' 30.47'' N, 100° 27' 42.32'' E | Mangrove forest turned into shrimp farm |
| V | 4° 56' 14.84'' N, 100° 28' 5.016'' E | Intertidal mudflat surrounded by residential, jetties and fishery activity |

(Beals et al., 2000), while distances from the release center and the size of the milky storks' foraging grounds were estimated using the GEPath software for areas less than 1000 hectares (Rahman, 2017).

### Statistical Analysis

One-way ANOVA with Tukey as post-hoc test was used to differentiate the number of visits made between foraging sites. All statistical tests were done at 95% level of significance using the Statistical Package for Social Science (SPSS) software version 17. Principal component analysis (PCA) using XLSTAT (version 2014.3.02) was also employed to classify recorded variables i.e. tree height, distant from release center, number of other waterbirds, number of milky storks and the size of the foraging areas into meaningful components to better understand the pattern of the milky storks' foraging activity. Variables that failed to meet the minimum criteria of having a primary factor loading of 0.4 or above were excluded from the analysis.

## RESULTS

There was a statistically significant difference between groups (i.e. number of visits recorded between available sites) as determined by one-way ANOVA ($F(4,50) = 36.33$, $p = 0.001$). The Tukey post-hoc test revealed that the number of visits made was highest in site I ($17.6 \pm 1.26$, $p = 0.001$). There were no statistically significant differences between visits made between site V and site IV ($12.5 \pm 4.0$ and $8.9 \pm 6.02$ respectively with $p = 0.865$) as well as site III and II ($3.0 \pm 0.7$ and $2.2 \pm 0.45$ respectively with $p = 0.988$). Based on the percentage of foraging visits made by the milky storks (Figure 2), the population's foraging activity as recorded was almost equal between the natural sites (i.e. intertidal mudflat and mangroves) and the shrimp farms between January and February 2013 (approximately 50% each). However, starting from March until May 2013, the population had moved to existing shrimp farms in Kuala Gula (Site III and Site IV). Later, between June and August 2013, the

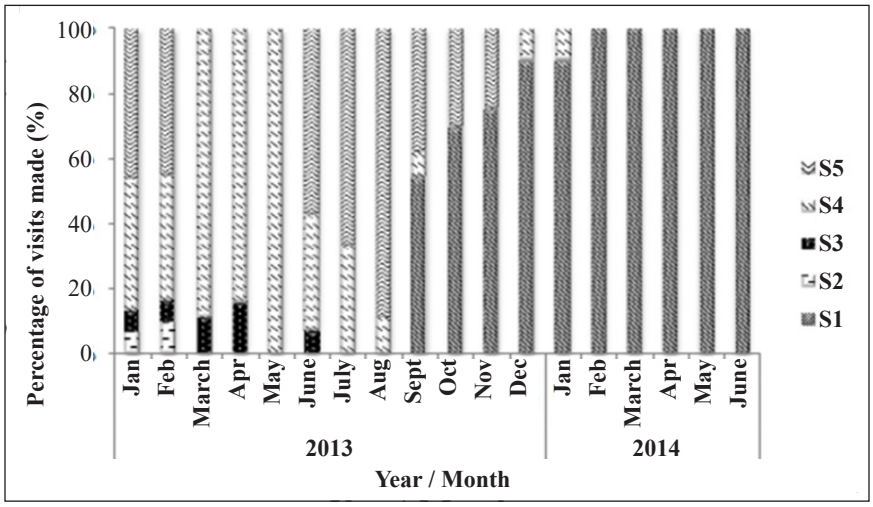


*Figure 2*. Percentage of foraging visits made by the milky stork in the study

population started to incorporate back the natural sites as their foraging area (57% – 88%), most notably because they were located in the same area as their nesting site. However, between September 2013 and June 2014, the storks had shifted most of their foraging activity to a newly built shrimp farm (Site I). In general, the size of their foraging area had increased up to 20% (30 ha) between 2013 and 2014.

As for their diet, the milky storks' prey predominantly consisted of brackish and coastal water species. In this study, we identified at least seven different species consumed by the reintroduced population. These include the *Mystus* sp., *Oreochromis* sp., *Penaeus* sp., *Periophthalmus minutus*, *Valamugil* sp. and also mollusks commonly found in the Kuala Gula coastal area. Figure 3 highlights the storks' diet which varies

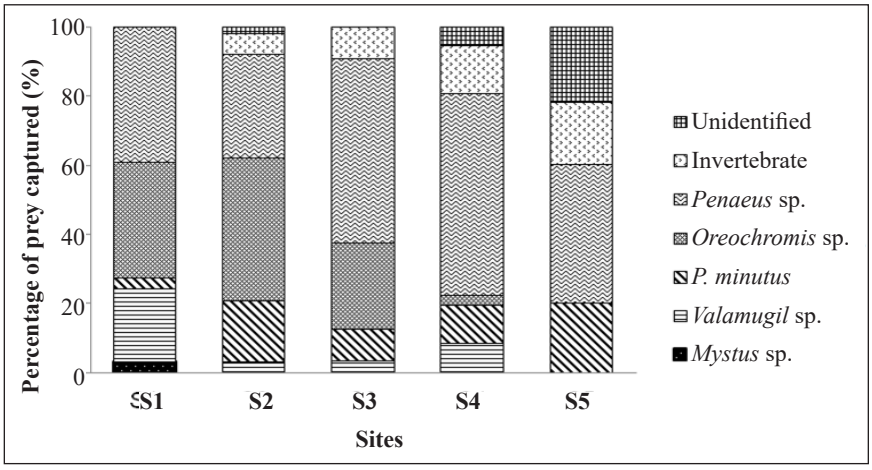


*Figure 3*. Percentage of preys captured by the storks in the study

according to available foraging sites. In general, the *Penaeus* sp. consumption was the highest (30 - 58%), followed by *Oreochromis* sp. (3 - 41%), *P. minutus* (3 - 20%), mollusk (6 – 18%), unidentified (2 - 22%), *Valamugil* sp. (3 - 21%), and *Mystus* sp. (2%). In addition, the consumption of *Periophthalmus minutus* by the milky stork is the first to be reported by this study.

For the principal component analysis results, the variables can be separated into two groups with the number of waterbirds foraging and area in one group, while tree height and distances from the release center in another (Table 2). Furthermore, the first two principal components account for 89.28% of the explained variances in this study. Axis 1 has strong positive loadings for the waterbirds population number, distances, tree height and area. This may reflect the general characteristic of the foraging habitats preferred by the re-introduced population. On the contrary, axis 2 has strong negative loading for the milky stork population and positive loading with tree height. This reflects the less foraged areas by the milky storks in Kuala Gula. The two components can be categorized into 'disturbed' and 'undisturbed' areas where the former primarily consists of reclaimed mangrove areas while the latter has no anthropogenic activity presence in it. Overall, the biplot (Figure 4) indicates that the foraging activity of the milky storks is more likely to be tied to the size of the foraging site.

## DISCUSSION

It is believed that in the early stages of establishment, birds tend to select high quality habitat that offers enhanced cover and foraging opportunities (Michele et al., 2010). In this study, the milky stork population had been relying on both natural and developed or disturbed areas for food and the latter was being utilized mostly towards the end of this study. This alternating or shifting of foraging area could be related to the changes in prey abundance and also the shrimp harvesting

Table 2
*Results of the principal analysis performed on the correlation matrix of the five variables describing the milky stork's foraging site characteristics*

| Variables | Abbreviation | PCA Axes | |
|---|---|---|---|
| | | 1 | 2 |
| Number of milky storks | Ms | 0.8 | |
| Number of other waterbirds | Other | 0.9 | |
| Distance from the release center (km) | Dist | 0.9 | |
| Tree height (m) | TH | | 0.7 |
| Size of foraging area (ha) | Area | 0.8 | |
| Eigenvalue | | 3.8 | 0.7 |
| Explained Variance | | 75.0 | 14.2 |
| Summation of Explained Variance | | 75.0 | 89.3 |

* Only the highly significant loading factors of the variables in the PCA axes are shown

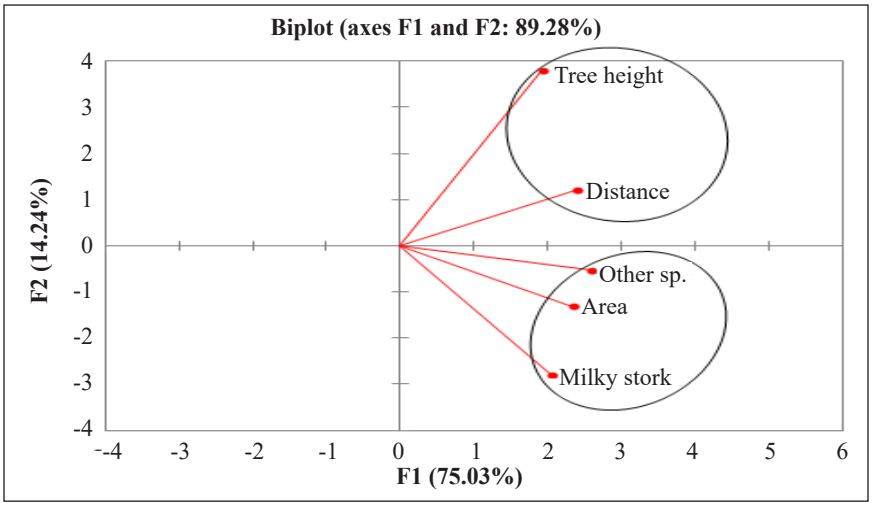


*Figure 4*. PCA results, the variables and their relationships as analyzed in the study

period in which the milky storks and other opportunist waterbirds often take advantage of (Marquiss, 1993). The development of a massive shrimp farm in Pulau Gula or Site 1 (approximately 340 ha) further led the milky storks to incorporate this area as their main foraging site (September 2013 - June 2014). Field observations suggest that the alteration of the landscape and its hydrology throughout the farms' development had trapped many wild fish and shrimps in the area. This presented an opportunity for both the milky storks and other waterbirds to feed on the prey easily.

The results from the principal component analysis are also in agreement with the shifting foraging pattern observed in the study. The milky storks have been increasingly incorporating existing and newly built shrimp farms as their foraging areas. However, the pattern becomes more prominent starting from the middle until the late period of this study (September 2013 to June 2014). The storks were given a fixed amount of fish (average of two kilograms daily) during their captivity and conditioning phases and the practice still continued post-release. Ismail et al. (2012) stated that the captive milky storks were not very active as they spent most of their time roosting. This was probably true due to the nature of the storks' enclosure which restricted the subjects' movement. However, as the milky storks were re-introduced into the wild, they became more active. Therefore, the amount of food given may have no longer been sufficient to support the increasing energy requirements of the free roaming storks. Thus, the storks' strategy of incorporating the shrimp farms could be a way for them to compensate for the extra energy required to sustain themselves (Kloskowski et al., 2009). Field observation in South Sumatera showed that the population had taken milkfish (*Chanos chanos*), elongate mudskipper (*Pseudapocryptes elongatus*), mullet fish and even giant mudskipper (*Periaptholomodon schlosserii*) as part of

their diet (Iqbal et al., 2009). However, the milky storks in Kuala Gula were only recorded to prey on *P. minutus* although several other species of the mudskipper were available. The shifting in foraging areas utilization by the milky storks showed that they relied heavily on shrimp farms for food. The high consumption of *Penaeus* sp. recorded (up to 58%) was in line with the high number of visits made to the shrimp farms as it made up the bulk of the population's diet. Other preys captured in lower percentages (21% and less) like the *Mystus* sp. and *Valamugil* sp. were mostly taken opportunistically as they were trapped in the canals during the farms' development and operation. In addition, some species remain separated spatially from the brackish and estuary communities following the changes in the area's hydrology. As for the unidentified food items, we believe that they consisted of decomposed plant materials that were taken by younger individuals between the ages of one and two years (based on the identification rings or tags on their legs).

Fundamentally, the study found that the milky stork population's foraging movement and pattern could be tied to: 1) the availability of aquaculture activity i.e. shrimp farms, and 2) the opportunistic strategy adopted by the population to support the increase in energy required by post-release activity. Accordingly, there are several benefits gained by the milky stork which include but are not limited to: a) utilizing the shrimp farms as a relief and also as an important complementary foraging area to the population (Navedo et al., 2015), b) the conversion of mangroves to build new shrimp farms has led to an increase in area for them to forage on, and c) the selection or utilization of existing and new resources could indicate favorable conditions for its long-term survival (Manly et al., 2002). For a major habitat shift to happen, it can either be the result of a negative factor i.e. the deterioration of a previous habitat (Morris & Dupuch, 2012) or a positive one i.e. having flexibility as a strategy to survive adverse conditions (Bystrom et al., 2003). In the case of the milky stork, both factors could contribute to the findings and need to be monitored in the long run as the long-term impact such a pattern may have upon the milky stork is unknown. In addition, potential conflict is bound to happen as the storks continue to forage in shrimp farms. Although the waterbirds in general are regarded as pest or threat to the aquaculture industry as they prey on aquaculture products, the losses are actually relatively low compared to other causes of mortality such as diseases, accidents, and poor water quality (Kushlan & Hafner, 2000). As such, there is a need to raise awareness and support from the public as well as owners of the local industry to help protect the endangered population (Ismail & Rahman, 2016).

The continuous input of pollutants from existing and new anthropogenic activities could also have a negative impact on the population. As shrimp farms in Malaysia are generally established along the coastal mangroves, a high concentration of such activity in Kuala Gula could result

in an increase of pollution input to the surrounding area (Rahman et al., 2013; Vandergeest, 2007). Intense shrimp farming activity has been shown to have dire consequence to wildlife and their habitats. According to Anantanasuwong (2001), massive pollution of the coastal waters at rates that far exceed the natural systems regenerative capacity occurs when shrimp farms dispose their untreated water during and after harvesting periods. As a result, sectors that are dependent on coastal resources including their inhabitants have suffered from the pollution. A recent study done by Rahman et al. (2017) showed that the milky stork population could potentially accumulate high levels of heavy metals through their diet obtained near the common water canals. As such, the current foraging pattern observed could expose the milky storks to higher levels of hazardous pollutants in the long run. The sediment in particular serves as a reservoir for the residues of many hazardous pollutants including drugs, heavy metals and other pollutants (Ismail & Ramli, 1997; Rahman et al., 2013; Sather et al., 2006; Weston, 2000). Under certain conditions, they can re-enter the aquatic environment causing negative influence to the food webs through bio-accumulation and bio-magnification processes. Therefore, further study is required to highlight and monitor the level of hazardous pollutants in the milky storks' foraging areas and how they could affect the population in the long run. This is important in order to understand the risk that could be faced by this endangered species as well as their future in Kuala Gula.

## CONCLUSION

It is concluded that the reintroduced milky storks are well adapted to the new habitat in Kuala Gula. However, the findings also suggest that anthropogenic activity and landscape-alteration have had both positive and negative influence on the milky storks' foraging activity. The positive influence includes the use of such an area as an alternative foraging site which acts as a relief during food scarcity or when higher energy demand is required. It also allows for easy monitoring and studies to be conducted in the wild due to its being highly accessible. On the negative side, the quality of the mudflats, intertidal areas and rivers in Kuala Gula would be compromised in the long run due to pollution inputs. This will have a negative impact on the re-introduction program and the milky storks' conservation in general. The continuous success of the re-introduction program will very much depend on the quality of Kuala Gula's natural habitat. Protecting key environmental components that help sustain the population in the long run as well as incorporating them in the future planning and development of Kuala Gula is a must. Therefore, the future biodiversity and conservation planning of a similar scale should be critically assessed and account for the conflicting interests that may arise i.e. between protecting the natural habitat for conservation purpose versus the need for aggressive economic development in the area.

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*Short Communication*

# Retrospective Identification of Bacterial Depository Revealed that *Streptococcus iniae* was Responsible for Some of the Streptococcosis Cases in Cultured Red Tilapia in Malaysia since 2006

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## ABSTRACT

This paper reports the identification of *Streptococcus iniae* from a large collection of isolates previously identified as *Streptococcus* sp., *Lactococcus lactis* subsp. *lactis* or *Leuconostoc* sp. A total of 204 bacterial isolates recovered either from the brain, eye, or kidney of red tilapias in previous disease outbreaks and disease monitoring in Malaysia from 2006 to 2008 were used. PCR identification revealed that 34 (16.7%) of the isolates were confirmed as *S. iniae*. Our records showed that *S. iniae*-infected fish exhibited lethargy, exophthalmia, and erratic swimming patterns. Pathological lesions including generalised congestion of the internal organs, splenic infarction with soft and oedematous brain. Histopathological examination revealed multifocal encephalitis as one of the major findings. However, 44% and 26.5% of the tilapias from which *S. iniae* was isolated did not manifest any clinical sign and pathological lesion, respectively. This study revealed that *S. iniae* was responsible for streptococcosis in cultured red tilapia in Malaysia since 2006.

*Keywords*: *Oreochromis* sp., red tilapia, streptococcosis, *Streptococcus iniae*

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## INTRODUCTION

The intensification of tilapia culture in this country has led to disease outbreaks, especially due to *Streptococcus* spp., which cause high mortality and severe economic losses to the industry (Zamri-Saad et al., 2014). To date, *Streptococcus agalactiae*, *S. iniae*, and *S. dysgalactiae* were identified as the main aetiological agents of streptococcosis (Costa et al., 2014; El-Aamri et al., 2010; Rahmatullah et al., 2017). In Malaysia, infection by *S. agalactiae* has been previously reported affecting red tilapia (Syuhada et al., 2020) and golden pompano (Amal et al., 2012).

*Streptococcus iniae* has been associated with several disease outbreaks in both freshwater and marine cultured fishes, such as hybrid tilapia (Al-Harbi, 2011), Nile tilapia (Shoemaker et al., 2001), red porgy (El-Aamri et al., 2010), hybrid striped bass (Shoemaker et al., 2001), seabass (Colorni et al., 2002), and Japanese flounder (Nguyen et al., 2002). However, infection by *S. iniae* in cultured red hybrid tilapia in Malaysia was only reported in 2017 (Rahmatullah et al., 2017).

From 2006 to 2008, *Streptococcus* outbreaks and fish disease screening revealed the responsibility of *S. agalactiae* in infecting red tilapia in various aquaculture sites in Peninsular Malaysia (Amal et al., 2010), but no identification of *S. iniae* from the diseased fish was made. However, in this study, we revealed that *S. iniae* was also actually responsible for streptococcosis in cultured tilapia in Malaysia as early as 2006, where it was previously identified as *Streptococcus* sp. or other bacteria species.

## MATERIALS AND METHODS

In our previous studies, between 2006 and 2008, samplings of cultured red tilapias were carried out for bacterial isolation following complaints of disease outbreaks from farmers and routine disease monitoring. The sampling sites covered three different types of water bodies including reservoir, river, and pond, which comprised of nine different locations in the north and east part of Peninsular Malaysia (Table 1). The fish clinical signs, external and internal abnormalities of the fish were observed and recorded before the bacterial isolation was made from the brain, eye, and kidney. Selected organs of the diseased fish such as skin, liver, spleen, brain, and kidney were also collected for histopathological analyses (Amal et al., 2010, 2015; Zamri-Saad et al., 2010).

Following the bacterial isolation and identification in the period of 2006 to 2008, besides *S. agalactiae*, a total of 204 Gram positive isolates were also identified either as *Streptococcus* sp., *Lactococcus lactis* subsp. *lactis*, and *Leuconostoc* sp. using API 20 Strep (bioMérieux, Marcy l'Etoile, France) (Table 1). In order to screen for *S. iniae*, all of the isolates were then retrieved from our collections, and subcultured onto tryptic soy agar (Merck, Darmstadt, Germany) with 5% goat's blood and incubated at 27°C for 24 h to 48 h. A single colony from each plate with pure bacterial growth was then selected and

inoculated into 10 mL brain heart infusion broth (Merck) before incubated in an orbital incubator at 27°C for 24 h at 150 rpm. DNA from the isolates was extracted using Wizard Genomic DNA Purification Kit (Promega, Madison, USA) as per manufacturer's protocol. The extracted DNA was optimized for concentrations ranging between 3.5 and 4.0 µg/µL prior to PCR amplification.

A published primers sequence for the targeted gene *lctO* of *S. iniae* was used (Mata et al., 2004). The nucleotide sequences for the forward and reverse direction were LOX-1 5'AAGGGGAAATCGCAAGTGCC3' and LOX-2 5'ATATCTGATTGGGCC GTCTAA3', respectively. The PCR assay was carried out based on the method described by Mata et al. (2004). For comparison and validation of the PCR technique, *S. iniae* strain ATCC® 29178™ (positive control), distilled water (negative control), and *S. agalactiae* strain ATCC® 27956™ (negative control) were included in each test.

## RESULTS

A total of 16.7% (34) of the isolates were successfully identified as *S. iniae*, following amplification of the 870 bp band in PCR methods. The positive control of *S. iniae* ATCC® 29178™, negative control of distilled water, and negative control of *S. agalactiae* ATCC® 27956™ were used to validate the methods revealing their respective expected results (Figure 1). All *S. iniae* isolated in this study showed β-haemolysis on blood agar.

*Streptococcus iniae* was detected from cultured red tilapia at six (67%) of the nine sampling sites. Most isolates originated from Terengganu (31 isolates; 91.2%), while the remaining two (5.8%) and one (2.9%) were from Kedah and Perlis, respectively. They were isolated either from the brain, eye or kidney of the fish that between 19.7 to 31.5 cm in length and 175 to 967 g of body weight (Table 1). Interestingly, it was found that larger tilapias seemed to be more susceptible to streptococcosis. Our previous data showed that all 34 isolates of *S. iniae* were earlier identified as *L. lactis* subsp. *lactis* (28 isolates; 82.4%), *Streptococcus* spp. (3 isolates; 8.8%), and *Leuconostoc* spp. (3 isolates; 8.8%) when using the API 20 Strep kit.

Database showed that approximately 44.1% of red tilapias from which *S. iniae* was isolated did not show any gross lesion and clinical sign, while 55.9% either showed corneal opacity, unilateral or bilateral exophthalmia, inflammation along the base of the pectorals region, ventral region, operculum, and erratic swimming patterns. Following post-mortem examination, 26.5% of the affected tilapias appeared normal, while the remaining 73.5% either showed congestion of gill, liver, spleen, and kidney, with soft and oedematous brain. Histopathological examination confirmed the generalised congestion of internal organs and splenic infarcts, while the brain revealed multifocal encephalitis and oedema (Figures 2 and 3).

Table 1
*Details of the identified* Streptococcus iniae *from cultured red tilapias in several sampling sites in Peninsular Malaysia*

| Water body | Sampling site | State | Year of isolation | No. of tested isolates | No. of positive isolates (%) | Organ | Infected fish | | API20 Strep identification (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Fish length Mean ± SD (cm) | Fish weight Mean ± SD (g) | |
| Reservoir | Pedu Lake | Kedah | 2006 | 55 | 2 (3.6) | E, K | 19.75 ± 2.47 | 175.00 ± 63.64 | *Streptococcus* sp. (100) |
| | Kenyir Lake | Terengganu | 2008 | 4 | 2 (50.0) | E | 28.25 ± 0.35 | 443.50 ± 20.51 | *Lactococcus lactis* subsp. *lactis* (100) |
| River | Kuala Kejir | Terengganu | 2008 | 3 | 0 (0.0) | - | - | - | - |
| | Pantai Ali | Terengganu | 2007-2008 | 15 | 2 (13.3) | B, E | 16.00 ± 6.36 | 125.50 ± 132.23 | *Lactococcus lactis* subsp. *lactis* (100) |
| | Beladau Selat | Terengganu | 2006-2007 | 64 | 24 (37.5) | B, E, K | 20.69 ± 3.99 | 224.71 ± 133.32 | *Lactococcus lactis* subsp. *lactis* (95.83) and *Leuconostoc* spp. (4.17) |
| | Beladau Kepong | Terengganu | 2006-2008 | 23 | 3 (13.0) | E | 20.50 ± 4.09 | 176.67 ± 82.02 | *Lactococcus lactis* subsp. *lactis* (33.3) and *Leuconostoc* spp. (66.6) |
| | Arau | Perlis | 2006 | 30 | 1 (3.3) | B | 31.50 | 967.00 | *Streptococcus* sp. (100) |
| | Kodiang | Kedah | 2008 | 5 | 0 (0.0) | - | - | - | - |
| Pond | Jitra | Kedah | 2006 | 5 | 0 (0.0) | - | - | - | - |
| Total | | | | 204 | 34 (16.7) | | | | |

*Note.* B = brain; E = eye; K = kidney

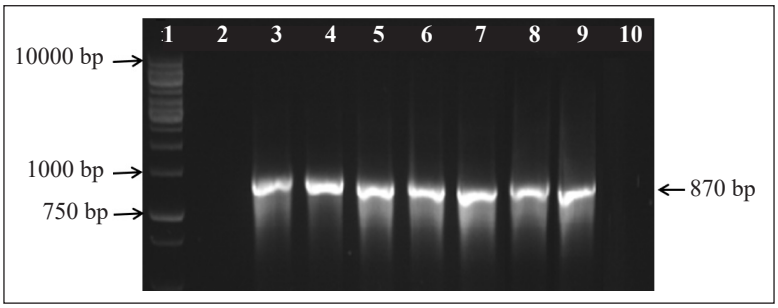
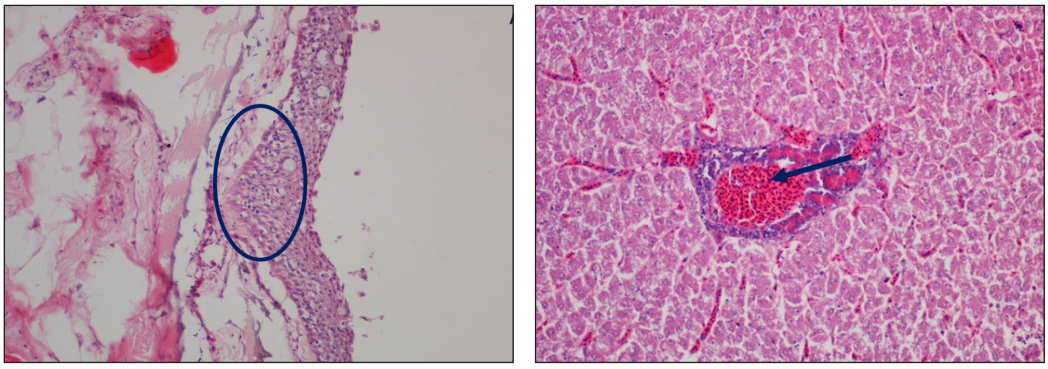
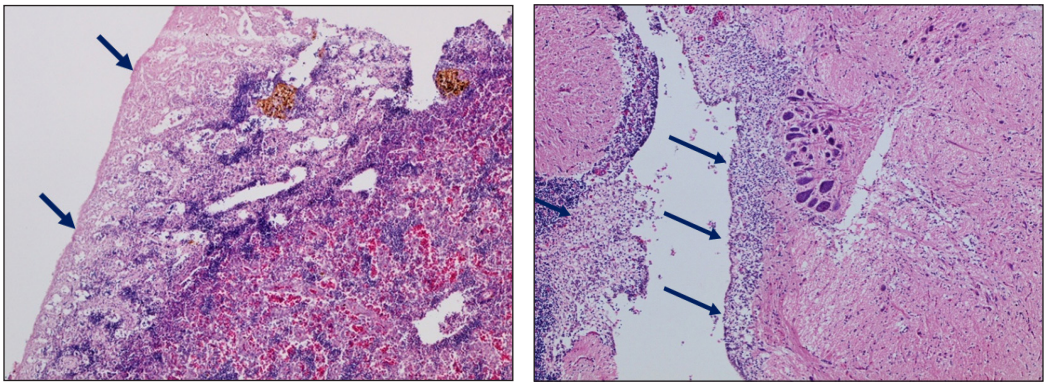



*Figure 1.* Agarose gel electrophoresis showing PCR amplification products generated by the LOX-1/LOX-2 primers at 870 bp. Lane 1: 10000 bp molecular size ladder; Lane 2: distilled water (negative control); Lane 3: *Streptococcus iniae* ATCC® 29178™ (positive control); Lane 4: TP3 (isolate from Pedu Lake); Lane 5: TCT1044 (isolate from Kenyir Lake); Lane 6: TSP299 (isolate from Pantai Ali); Lane 7: TSB274 (isolate from Beladau Selat); Lane 8: TSK682 (isolate from Beladau Kepong); Lane 9: TA127 (isolate from Arau); Lane 10: *Streptococcus agalactiae* ATCC® 27956™ (negative control)



(a) (b)

*Figure 2*. (a) Skin section showing inflamed epithelial layer with infiltration of inflammatory cells (oval). H.E., obj. 100×; (b) Liver section showing congested portal vein (arrow). H.E., obj. 400×



(a) (b)

*Figure 3*. (a) Infarct in spleen (arrow). H.E., obj. 200×; (b) Brain section showing extensive encephalitis (arrow). H.E., obj. 100×

## DISCUSSION

The inability of API 20 Strep to correctly identify *S. iniae* has been previously described (Al-Harbi, 2011; El-Aamri et al., 2010; Roach et al., 2006). Previous studies reported that biochemical analyses using API 20 Strep misidentified *S. iniae* as *S. dysgalactiae* subsp. *equisimilis* (El-Aamri et al., 2010; Lau et al., 2003; Suanyuk et al., 2010). However, in this study, *S. iniae* were previously identified as *Streptococcus* spp., *L. lactis* subsp. *lactis*, and *Leuconostoc* spp., which we believed to be due to misinterpretation on the reading of the biochemical tests based on the visual interpretation, as raised by Gomes et al. (2007). Other commercial test kits, such as BioMèrieux Vitek, MicroScan WalkAway System (Facklam et al., 2005), and ATB Expression System (Lau et al., 2003) were also unable to identify *S. iniae*. Due to this limitation, specific PCR primer sequences have been developed as a useful alternative approach for the rapid and accurate identification of *S. iniae*, such as the 16S rRNA gene, the 16S–23S rRNA gene intergenic spacer region, the chaperonin HSP60, and the *lctO* gene (Berridge et al., 1998; Goh et al., 1998; Mata et al., 2004; Roach et al., 2006).

In this study, we successfully identified *S. iniae* from cultured red tilapia in Malaysia by PCR based on the *lctO* gene. Utilization of this primer sets has also successfully identified *S. iniae* from several species of fish in different regions (Al-Harbi, 2011; Lee & Park, 2014; Suanyuk et al., 2010). Affected tilapia in this study showed similar clinical signs as previous reports (Chen et al., 2007; Rahmatullah et al., 2017). However, there were evidences of asymptomatic carriers, as observed in striped piggy and variegated lizardfish (Colorni et al., 2002). They showed neither clinical signs nor pathological changes, and could be a source of infection as observed in *S. agalactiae* (Amal & Zamri-Saad, 2011).

## CONCLUSION

Future bacterial identification should include the molecular and sequencing analysis for better accuracy of the results. Moreover, this study revealed that *S. iniae* was actually responsible for streptococcosis in cultured red tilapia in Malaysia since 2006.

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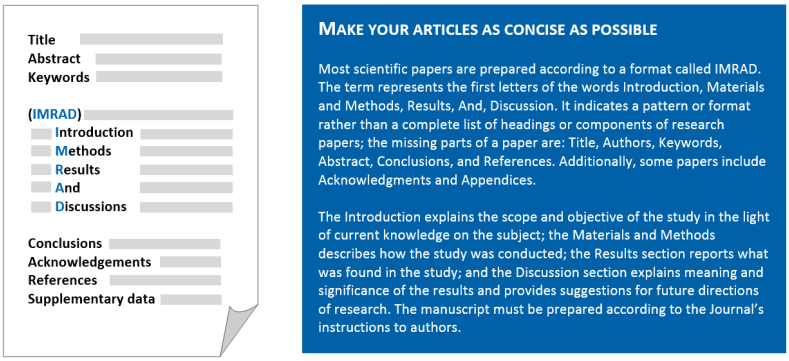


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