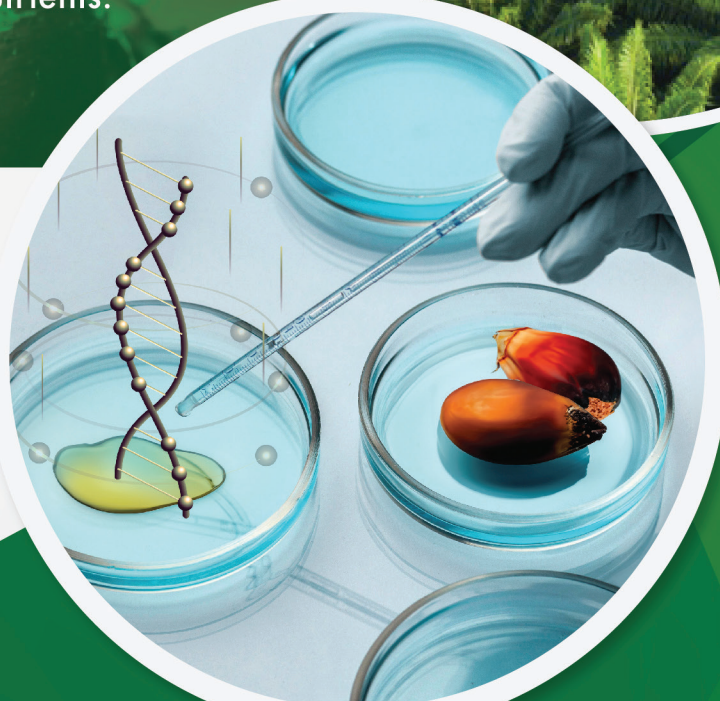


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REVIEW ARTICLE

- The Environmental and Health Sustainability Challenges of Malaysian Palm Oil in the European Union
- *In Vitro* Studies on Palm Phytonutrients: A Nutrigenomic Review



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THE ENVIRONMENTAL AND HEALTH SUSTAINABILITY CHALLENGES OF MALAYSIAN PALM OIL IN THE EUROPEAN UNION

LAKSHMY NAIDU¹; RAVICHANDRAN MOORTHY^{1*} and MOHD IKBAL MOHD HUDA¹

ABSTRACT

The palm oil industry in Malaysia is an essential source of export revenue for the nation. As demand for palm oil grows in the international market, the industry has been experiencing mounting pressure to ensure sustainable development. The necessity for sustainable palm oil is amplified with European Union's requirement for certified and sustainably produced palm oil to enter its market. The commodity is also inundated with anti-palm oil campaigns, which emphasise allegations of unsustainable practices. Collectively, these challenges pose a formidable impact on the palm oil trade. However, there are insufficient studies that review the existing literature on the challenges related to palm oil sustainability. Hence, this article presents a review on the environmental and health sustainability concerns the Malaysian palm oil sector faces when trading in the European market. The findings of the article suggest the development of strategies by multi-stakeholders to improve commodity competitiveness in the international market. One strategy proposed in this article is to engage the media and Non-Governmental Organisations in developing effective communication mechanisms to disseminate a more balanced perspective of the commodity. A positive voice on the existing sustainability measures could help shape an unbiased public opinion on palm oil production, trade and consumption.

Keywords: environment, European Union, health, Malaysian palm oil, sustainability.

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INTRODUCTION

The need to produce renewable energy, reduce greenhouse gas (GHG) emissions and mitigate climate change effects has contributed to the rise in global demand for oilseed crops as an alternative to fossil fuel (Jaradat, 2016). Being a high-value seed oil, oil palm cultivation has a significant role in agricultural industries and international trade. As one of the major sources of oils and fats in the

world, palm oil is a highly efficient vegetable oil which is attributed to its high yield per hectare and low land area requirement as the crop produces an average of 4 t of oil per hectare per year (Morley, 2015; Rival and Levang, 2014). The wide-ranging qualities of palm oil such as long shelf life of 12 months minimum; stability in high temperatures of 175°C-185°C; high oxidative stability of 72 hr; odourless feature; easy conversion to various derivatives and fractions such as palm olein and palm stearin, glycerol, fatty acids and fatty alcohols; competitively priced and cost-effective due to its high productivity; and rich source of phytonutrients has increased its versatility and adaptability for the application in various food and non-food industries. These characteristics have led

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palm oil to be known as the most produced and consumed vegetable oil in the world since almost 50% of items in a supermarket contain palm oil (Hansen *et al.*, 2015; Oosterveer, 2015).

The growing demand for palm oil in the international market has resulted in the expansion of oil palm plantations in Malaysia. As the second-largest exporter of palm oil, the oil palm plantation area in Malaysia has reached 5.87 million hectares, supplying 34.3% of the total palm oil trade and constituting 18.3% (17.37 million tonnes) of the global oils and fats industry in 2020 (Parveez *et al.*, 2021). In terms of national income, the export revenue from palm oil and its related products contributed RM108.52 billion in 2021 (Parveez *et al.*, 2022). As an export-oriented agricultural commodity, the oilseed crop can meet increasing demand as the world population is expected to increase to nine billion by 2050 (MPOC, 2021a). However, the government's policy on the limitation of national oil palm plantation area to 6.50 million hectares has steered concerns on fully optimising land usage to produce a sufficient supply of high-quality palm oil through sustainable agricultural practices (Mohan *et al.*, 2020).

Amidst ongoing scientific research to improve the sustainability of the overall palm oil supply chain, the oilseed crop is controversial among policymakers, scientists, academicians, Non-Governmental Organisations (NGOs) and the general public in Europe. As one of the forest risk commodities, oil palm cultivation is often associated with environmental damage such as loss of tropical rainforest, peatland draining and burning, destruction of biodiversity, increase in GHG emissions and carbon footprint, water pollution, and loss of flood regulators (Austin *et al.*, 2017; Corciolani *et al.*, 2019; IIED, 2017; Kiew *et al.*, 2020; Lupascu *et al.*, 2020; Meijaard *et al.*, 2020; Moorthy and Jeyabalan, 2012; Moorthy *et al.*, 2011; Saswattecha *et al.*, 2017). The degradation of the natural ecosystem, which is perceived to be linked to oil palm cultivation, has placed the commodity in a bad light.

Apart from ecological impacts, issues on the effect of palm oil on public health have continued to be present in the past decades. Palm oil contaminants, such as 3-monochloropropane-1,2-diol esters (3-MCPDE) and glycidyl esters (GE), can pose possible risks to human health (EFSA, 2016; Gao *et al.*, 2020; O'Brien, 2008; Sulin *et al.*, 2020). Moreover, mineral oil hydrocarbon (MOH) contaminant such as mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH) found in oil palm fruits during processing at mills, has carcinogenic and genotoxic constituents, which can cause harmful consequences on overall health (Ahmad *et al.*, 2019; Sdrigotti *et al.*, 2021). In addition to its association

with cancer, high consumption of palm oil has been related to obesity, high blood cholesterol and cardiovascular diseases (Chapman, 2017; Chong and Ng, 1991; Corciolani *et al.*, 2019; McNamara, 2010). Based on the narrative, the concerns over the safety of palm oil in the food sector continue to dominate the discourse on public health.

The negative image of palm oil in the environmental and public health aspects has raised interest in the sustainability of the commodity in the European Union (EU). Despite being a suitable option for the transition from fossil fuel to renewable energy sources, palm oil is highly contested and regulated in the region. Beginning in 2015, the EU has established certified and sustainable palm oil standards as part of its importation requirements to manifest an entirely sustainable palm oil supply chain in Europe by 2020 (EU, 2021). Furthermore, the European Parliament resolution on palm oil and deforestation has emphasised on the development of minimum sustainability standards for imported palm oil. The detrimental effects of palm oil production have been taken into consideration in the revised Renewable Energy Directive (RED) II. The restriction on the use of the crop for the production of biofuels is further implemented in the delegated regulation that focuses mainly on the determination of Indirect Land Use Change (ILUC)-risk and on the certification of low ILUC-risk biofuels (EC, 2021; Kannan *et al.*, 2021). ILUC occurs when agricultural land originally intended for food and feed markets is converted for biofuel production. The competing demand for certain feedstock to be used as fuel has resulted in more land being cleared. A feedstock is categorised as high ILUC risk if the significant expansion of the production area is onto a high carbon stock area (Subramaniam *et al.*, 2019). Based on the delegated regulation, all biofuel feedstocks categorised as high ILUC-risk, not only limited to palm oil, are to be phased out by 2030 (Hinkes, 2020).

Contrary to the legislative approach for palm oil-based biodiesel, the EU has set only general product import and specific safety requirements for food and non-food processing purposes. For example, the European Commission (EC) implemented maximum limits on 3-monochloropropane-1,2-diol esters (3-MCPDE) and GE in edible oils and fats, while the European Food Safety Authority (EFSA) stated that the maximum concentration of 3-MCPDE and GE permitted for human consumption should be limited to 0.8 µg / kg of body weight / day (Beekman *et al.*, 2021; Restiawaty *et al.*, 2021). In adherence to good governance, MOH contamination in crude palm oil for food processing is administered through the Hazard Analysis Critical Control Point (HACCP) system (Ahmad *et al.*, 2019). The imposition of regulations and standards on

sustainability may undermine the marketability of palm oil vis-à-vis other vegetable oils and fats in the transportation and food industry.

There is increased compulsion for Malaysia's palm oil-related sectors to meet the sustainability requirements to trade competitively in the European market. Hence, this article aims to discuss the relevant literature by examining the growing body of evidence on the environmental and health perspective of the sustainable palm oil industry in Malaysia. The EU was chosen for the article as the regional bloc is the third largest importer of palm oil from Malaysia (Parveez *et al.*, 2020). As one of the important export markets, the EU imported 1 639 059 t of palm oil in 2021 (MPOB, 2022). Notably, Malaysian palm oil in the EU has been subjected to the stringent requirement for sustainable production in the past decades (Salleh *et al.*, 2021). The concerns about the sustainability of the industry can potentially affect the marketability of palm oil products in the EU. More importantly, this article provides an analysis of peer-reviewed literature about environmental and health issues on palm oil development and consumption, particularly in the EU. The result of this article postulates a better understanding of the underlying realities that would be beneficial to policymakers in formulating strategies and researchers in delivering academic discourse.

This article is structured into several sections. The following section discusses the materials and methods used for the writing of this article. Section 3 synthesises the literature, and elucidates the main findings and suggestions on strategies to be implemented. The last section summarises the article, states the limitation of the study and provides suggestions for future research.

MATERIALS AND METHODS

This article investigates the environmental and health sustainability issues of the Malaysian palm oil industry in the EU. Using the PICO approach by Lockwood (2015), the article consists of three main aspects, which are EU (Population), environmental and health sustainability challenges (Interest) and palm oil in the EU market (Context). Hence, the main research question for this paper is "What are the environmental and health sustainability challenges of Malaysian palm oil in entering the European Union?"

Three main keywords were identified based on the research question: *Palm oil*, *sustainable* and *European Union*. The searching process to locate suitable and relevant articles was conducted in the Scopus and Web of Science database. Only journal articles and review articles were selected for the purpose of this study. In addition, the screening

process is limited to include articles published within a period of 10 years (2011 to 2021) to discuss the latest progress in the industry. Articles published in English were selected for this study since the available literature on the subject-matter in English is adequate for academic analysis, as well as to reduce the cost and time involved to translate articles written in other languages. Following the research question, articles that focus on related areas of environmental and public health involving palm oil in Malaysia and EU were selected for this article.

RESULTS AND DISCUSSION

Based on the literature extracted from the Scopus and Web of Science database, two main sections were identified, namely environmental sustainability and health sustainability. These two sections were further categorised into four and two sub-sections, respectively. The sub-sections are the evolution of EU biofuel policies, categorisation and projection of ILUC, consideration of yield production and GHG reduction, and sustainability certification under the environmental sustainability section, while the section on health sustainability consists of the positioning of palm oil as a potential health risk and nutritional benefits of palm oil consumption as the sub-section. The results, as elaborated according to the sections and sub-sections, provided the answer to the research question.

Environmental Sustainability

The discourse on the environmental sustainability of palm oil has enormously intensified among policymakers at government institutions for several decades. The political debate on palm oil as feedstock for biodiesel production in the EU member states continues to dominate the discussion on the environmental impact of (un)sustainable palm oil. The literature review, as discussed in the following sub-sections, elucidates the discussion.

Evolution of EU biofuel policies. The biofuel policy developed by the EU is significant due to its role as an alternative to fossil fuel that decarbonises the transport sector to contribute to mitigating climate change effects (Bockey, 2019). Initially, biofuel was encouraged as transport fuel through Biofuel Directive in 2003 with non-binding targets of 2.00% in 2005 and 5.75% in 2010 (Oosterveer, 2020). However, the voluntary targets did not produce the intended policy outcome, and as such, the Renewable Energy Directive (2009/28/EC – RED I) was formulated with a binding target of a minimum 10% share of renewable energy as a transport fuel for each member state. Some of the sustainability criteria of the directive are biofuel products must be able to

decrease GHG emissions by at least 35% compared to fossil fuels, not produced from feedstock gained from land with high carbon stock such as wetland, peatland and forest land, adherence to EU standards of good agricultural practices, and must be gained from land that was already planted before January 2008 (Bockey, 2019; Stattman *et al.*, 2018). Notably, non-compliance with these requirements does not prohibit biofuel resources and crops into the EU market, but it cannot be accounted for as part of the target set by RED I.

However, RED I biofuel policy has been challenged by various interest groups from EU member states. The criticism ranges from the absence of GHG emissions from ILUC due to the cultivation of feedstock for biofuel production, debates on using agricultural land for fuel instead of food as well as the preference for imported feedstock rather than domestically produced vegetable oils such as rapeseed and sunflower for the production of biofuel (Oosterveer, 2020). The target of this debate is palm oil-based biodiesel due to its perceived association with the global loss of tropical rainforests (Pehnelt and Vietze, 2013). As such, the EU Parliament (EP) has passed a resolution (2016/2222(INI)) on the restrictions on palm oil imports for use as biodiesel with the intention to ban the commodity beginning 2020s and completely phasing out by 2030 (Goggin and Murphy, 2018; Hinkes, 2020; Kamaruddin, 2020). This resolution was formulated as part of the Renewable Energy Directive (2018/2001/EC – RED II), which increased the share of renewable energy in the transport sector to 14% in 2030 (Bockey, 2019).

While existing sustainability criteria are maintained, the renewed policy framework introduced more stringent sustainability requirements for future adherence among member states. These standards are a reduction in the target of biofuels produced from food crops to a maximum of 7% from an initial 10%, GHG savings set at 65% after 2021 and most controversially, the

introduction of high and low ILUC-risk for biofuel crops (Oosterveer, 2020; Sumfleth *et al.*, 2020). Based on the Delegated Regulation (EU) 2019/807 of RED II, palm oil has been categorised as a high ILUC-risk biofuel, whereby the cultivation of feedstock for biodiesel results in a significant expansion of the production area into high carbon stock land (Hinkes, 2020; Limenta, 2020; Naidu and Moorthy, 2021; Sumfleth *et al.*, 2020). As such, EU Members will not be able to consider palm oil-based biodiesel as a renewable transport fuel target (Limenta, 2020). In addition, palm oil imports to the EU will be limited to 2019 levels with the ultimate aim to phase out by 2030 (Bockey, 2019; Limenta, 2020; Moenardy *et al.*, 2021). *Figure 1* below illustrates the evolution of policies pertaining to biofuel in the EU.

The move to eliminate palm oil-based biodiesel in the European market can be construed as a discriminatory regulation that would inevitably increase the demand for less environmentally friendly crops with a higher land requirement such as soybean, rapeseed and sunflower (Hinkes, 2020; Subramaniam *et al.*, 2019). By specifically classifying palm oil as a high ILUC-risk biofuel, the policy has limited the sustainability criteria to a central determining standard, which applies to all biofuel crops (Kushairi *et al.*, 2019). Although the regulation intends to improve the sustainability of palm oil entering the European market, the diligence requirement will create an operational burden and increase transaction costs for growers and traders (Dolle, 2017; Partiti, 2020). In essence, the EP resolution and RED II, together with the delegated regulation, have resulted in a political dispute between the EU and palm oil-producing countries, such as Malaysia as these regulations impose restrictions on palm oil trade and use in the European market.

As a strategic trading partner for Malaysia, the changes in EU policies and regulations on the import of palm oil would affect the palm oil industry. The growing demand for palm oil

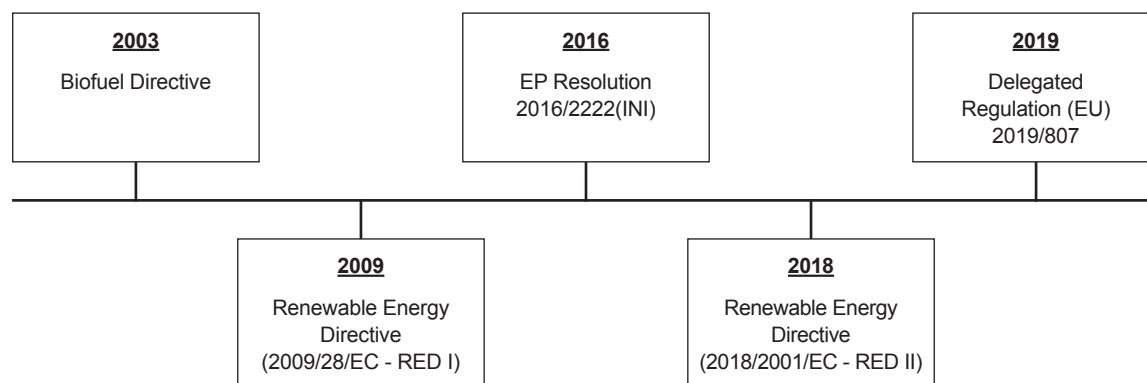


Figure 1. Evolution of biofuel policies in the EU.

as feedstock for biodiesel production may be negatively affected by the political interventions that promote the environmental agenda. More critically, the imposed regulation on palm oil import has raised concerns about its compatibility with World Trade Organization (WTO) law (Mayr *et al.*, 2021).

Categorisation and projection of ILUC. Despite the lack of concrete scientific evidence, the ILUC was included in the EU's revised policy on renewable energy. The inclusion of ILUC will result in additional GHG emissions for biofuel, which may be higher than fossil fuel (Subramaniam *et al.*, 2019). This has led to intense debates on the inconsistencies of the relatively new concept of ILUC compared to other well-developed and verifiable standards such as Life Cycle Assessment (LCA) and carbon footprint used to quantify carbon emission (Rival *et al.*, 2016). As a result, the inclusion of ILUC into biofuel policy may be viewed as politically driven and not entirely from the environmental perspective. This is evident as the expansion of oil palm cultivation to high carbon stock land was calculated at 45%, much higher than the least-productive and more environmentally damaging soy plantation which was set at 9% (Moenardy *et al.*, 2021; Oosterveer, 2020; Subramaniam *et al.*, 2019). As such, the conversion of high carbon stock areas for soybean plantations may cause more harm to the environment due to the crop's comparatively high land requirement and low yield production (Bockey, 2019; Hinrichsen, 2016; Subramaniam *et al.*, 2019). Other forest risk commodities such as beef production account for nearly 60% of deforestation, which makes it the primary driver of forest loss (Henders *et al.*, 2015). This has raised concerns that the indicator may be scientifically biased as environmental impacts associated with other vegetable oil feedstocks and forest risk commodities are not considered (Henders *et al.*, 2015; Kamaruddin, 2020).

Several interpretations of ILUC may be perceived as prejudice to countries producing biofuel feedstock. Some of the contestation regarding ILUC is the absence of a regional approach in categorising feedstock, lack of consideration for expansion into land with no high carbon stock, no consideration for loss of forest before the year 2008, and no regard for yield performance and land utilisation of biofuel crop (Subramaniam *et al.*, 2019). More notably, the ILUC projections are based on historical data on deforestation. Current development and improvement in technologies and practices are not considered in the projection of ILUC. For example, crop and livestock integration at oil palm plantations can potentially prevent ILUC by reducing further tropical forest loss (Azhar *et al.*, 2021). Thus, these shortcomings of

ILUC in EU biofuel policies have undermined sustainability initiatives and good agricultural practices implemented by the Malaysian palm oil sector. Some of the current measures undertaken are wastewater management, usage of organic fertilisers, utilisation of palm biomass and suitable planting methods (Aziz *et al.*, 2021; Kushairi *et al.*, 2018).

Consideration of yield production and GHG reduction. As one of the most productive oilseed crops, palm oil has an economic lifespan of 25 to 30 years. After three years of planting, the oil palm trees start to produce fresh fruit bunches (FFB) and reach their maximum yield by age 12 until 15 before decreasing (Nambiappan *et al.*, 2018). Compared to other biofuel crops, oil palm is 5.56 times more productive than rapeseed, 10.53 times more productive than soybean and 7.84 more productive than sunflower (Subramaniam *et al.*, 2019). This translates to palm producing around 4.00 to 6.00 t/ha/yr of oil as compared to rapeseed (1.50-2.50), soybean (0.38) and sunflower (0.51) (Bockey, 2019; Hinrichsen, 2016; Subramaniam *et al.*, 2019).

Due to the low yield performance of the other crops, the land required to produce one million tonnes of oil for soybean crop is 2.6325 million hectares, rapeseed crop (1.3900 million hectares) and sunflower crop (1.9600 million hectares). However, palm crop requires only 0.2500 million hectares of land to produce one million tonnes of oil (Subramaniam *et al.*, 2019). Hence, the productivity level of the crop contributes toward palm oil being a cost-effective feedstock for biodiesel production compared to domestically produced vegetable oil (Mat Yasin *et al.*, 2017; Santeramo *et al.*, 2021). Despite palm oil being a more effective oil-bearing crop in terms of yield performance and land usage, the commodity has been categorised as high ILUC-risk while the other feedstocks are classified as low ILUC-risk biofuel. Ultimately, the categorisation may subsequently lead to more land conversion that would increase GHG emissions.

For example, studies have shown that substituting palm with rapeseed crops would result in a 34.0% increase in agricultural land utilisation (Konstantas *et al.*, 2019). In fact, the research found that the potential GHG emissions savings of palm oil biodiesel were above the 35.0% target set, which is between 38.5% and 41.0%, as transport fuel depending on the fossil fuel comparator (Pehnelt and Vietze, 2013). Moreover, findings from another research suggest inconsistency in the existing calculation methodology and insufficiency in the default value for calculating GHG emissions from palm oil-based biodiesel (Stichnothe *et al.*, 2014). Evidence has also indicated that biofuel crops can enhance biodiversity in agricultural land by using wildlife-friendly practices (Verdade *et al.*, 2015). In

addition to GHG emissions, methane gas emitted from palm oil mill effluent can be trapped to operate palm oil mills, as part of the industry effort to reduce carbon footprint (Nambiappan *et al.*, 2018).

This usage of ILUC as sustainability criteria without consideration of other factors and its associated consequences can be detrimental to the overall ecological system. It could potentially negate the intended emission savings of biofuel, which was one of the sustainability criteria and fundamentals of EU environmental policies. The EU member states may view the criteria as restricting their capability to attain their binding GHG emission reduction target, while biofuel feedstock-producing countries such as Malaysia may construe the criteria as a form of trade discrimination. Given the current situation, one possible measure to effectively limit land-use change and reduce GHG emissions is through a combination of supply and demand-side policies formulated in the producer and consumer countries, respectively (Henders *et al.*, 2015; Verdade *et al.*, 2015).

Sustainability certification. The emergence of controversies that undermine palm oil's legitimacy has led to a growing interest in the development of sustainable production standards. The establishment of transnational sustainability certification (TSC) such as the International Sustainability and Carbon Certification (ISCC) and Roundtable on Sustainable Palm Oil (RSPO) compels palm oil-producing countries such as Malaysia to implement good agricultural practices, in accordance with international sustainability standards, as a pre-requisite to trading in the European market. The good agricultural practices are undertaken such as wastewater management, usage of organic fertilisers, utilisation of palm biomass and suitable planting methods can position Malaysian palm oil in a more balanced perspective among European member states (Aziz *et al.*, 2021; Kushairi *et al.*, 2018; Stattman *et al.*, 2018). The existing sustainability certification frameworks can elucidate the dynamics and complexity of the global governance mechanism for palm oil.

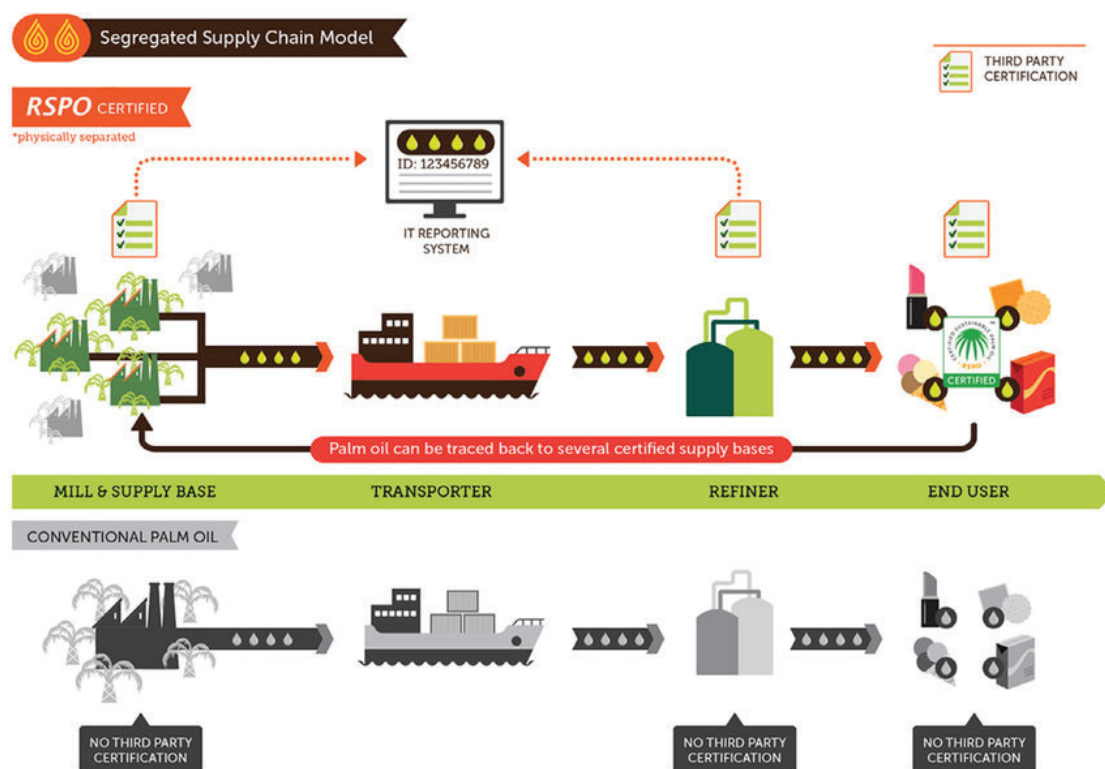
As a private voluntary sustainability scheme, the ISCC covers biofuel production's environmental and social aspects such as climate mitigation, biodiversity conservation, improved labour conditions, and minimum wages on biofuel farms (Stattman *et al.*, 2018). Since palm oil is an imported feedstock to produce biodiesel, the commodity needs to be certified under ISCC to guarantee its sustainability (Stattman *et al.*, 2018). The ISCC is based on the criteria and assessment of sustainability as stipulated in EU RED, which ultimately dictates the biofuel market (Oosterveer, 2020). Therefore, the ISCC certification serves as proof of compliance with sustainable biofuel production requirements

and thus, enables palm oil industry players to trade in the European biofuel market (Hinkes, 2020).

Contrary to the ISCC, RSPO serves as a governance framework for the production, trade and use of certified and sustainable palm oil (CSPO) in the European region for food and non-food processing sectors (Morley, 2015; Naidu and Moorthy, 2021; Oosterveer, 2020). This voluntary certification scheme was initiated in 2004 and led by private sectors using a multi-stakeholder approach which comprises palm oil industry players and NGOs. The sustainability certification aims to promote the economic, social and environmental sustainability of palm oil through open dialogue among stakeholders (Morley, 2015). There are four supply chain models in the RSPO certification scheme – identity preserved, segregated, mass balance, and book and claim. Ultimately, the classification of sustainable and unsustainable palm oil will minimise the latter's import into the EU market (Dolle, 2017; Gordeeva, 2017). Figure 2 presents an example of the RSPO segregated palm oil supply chain model compared to a conventional palm oil supply chain.

Despite the certification being widely accepted and recognised in the European region, there has been criticism against the sustainability scheme. Concerns such as lack of legitimacy and balance in its membership, unfair distribution of costs and benefits between downstream sectors and upstream sectors, operational barriers and financial constraint of certification among smallholders, lack of validity and respect toward national regulatory and sovereignty, complexity in the traceability and transparency of sourcing sustainable palm oil in the supply chain, and conflicting priorities for sustainable development between palm oil-consuming countries and palm oil-producing countries have created resistance towards the TSC initiative (Aziz *et al.*, 2021; Gassler and Spiller, 2018; Rival *et al.*, 2016; Ruysschaert *et al.*, 2019; Vogelpohl, 2021). In fact, there appears to be inconsistency in benchmarking standards among the TSC, particularly on biodiversity protection and conservation (Englund and Berndes, 2015).

As a response, the Malaysian Sustainable Palm Oil (MSPO) Standards were established in 2013 and the MSPO Certification was implemented voluntarily in 2015 as a mandatory state-led certification standard to monitor the development of sustainable palm oil supply chain (Kamaruddin, 2020). As a territorial representation of sustainability governance, the principles and criteria of the MSPO standard reframe the interpretation of sustainability according to national needs. Factors such as economic development requirements, domestic regulatory systems, prevalent state-industry relations, and existing practices and institutions are considered for the development of responsible practices from the environmental, social, health and safety, and



Source: Goggin and Murphy (2018).

Figure 2. An example of the RSPO segregated palm oil supply chain model compared to a conventional palm oil supply chain.

legal perspectives (Vogelpohl, 2021). Therefore, the marked difference in the interpretation of sustainability has led palm oil to be construed and promoted as sustainable vegetable oil due to its efficiency and productivity (Hinkes, 2020). However, the MSPO still depends on transnational institutions to access the European market to stay competitive internationally. The limitations, which can be viewed as technical and political constraint, exist since the TSC governance mechanism continues to shape global environmental governance and trade regulations in Europe using market and regulatory mechanism.

Health Sustainability

The sustainability challenges of palm oil are not only restricted to the production and trade of the commodity as biodiesel feedstock. The sustainability concerns associated with the health dimension of palm oil has inundated agri-food industry players and stakeholders in the supply chain since the last few decades. Studies have demonstrated that the purchasing and consumption of palm oil in food products have posed challenges in the public domain, as discussed in the following sub-sections.

Positioning of palm oil as potential health risk. Apart from governmental regulations, palm oil and its wide-ranging uses in consumer products

have been a boycott target. NGOs and private organisations have advocated the boycotting approach to limit the use of palm oil for non-biofuel purposes. Some of the strategies implemented are avoiding the usage and purchase of products containing palm oil, the usage of “no palm oil” labels, promoting the use of certified and sustainably produced palm oil through product labels, and organising palm oil-free campaigns (Naidu and Moorthy, 2021; Oosterveer, 2020). Such approaches reflect that the best interest of the consumers is best protected when products containing palm oil are shunned. The assertion is made based on the claim that palm oil has adverse effects on consumer health and the environment. However, these claims are not supported by scientific evidence (Uthaya *et al.*, 2015).

In particular, the labelling of palm oil and its associated derivatives as well as fractions in the list of product ingredients, as per Regulation 1169/2011 on the Provision of Food Information to Consumers and Article 18 of Regulation 178/2002, can be perceived as aiming at direct consumer engagement through continuous awareness (Nambiappan *et al.*, 2018; Oosterveer, 2020; Rival *et al.*, 2016). These regulations are pivotal in regards to food labelling within the EU whereby it is mandatory to specify the origin of all refined vegetable oils and fats in the products. The usage of labels which details the origin of vegetable oil or fat demonstrates the

regional trading bloc enforcement of importing only traceable sustainably sourced palm oils into its many downstream market sectors (Goggin and Murphy, 2018). Nevertheless, these approaches adopted among consumers in the EU market may lead to a constraint in oil palm developments in producing countries such as Malaysia.

Since consumers can decide to purchase the product based on the list of ingredients, manufacturers are constrained to select CSPO or replace palm oil with less controversial vegetable oil to dodge criticism from consumers and NGOs (Ruggeri and Samoggia, 2018). As such, the increased visibility of palm oil through comprehensive product labels may impact the decision-making process, especially among consumers who are health and environmentally conscious. Hence, consumers will have a selection of possible options to choose from that would meet their requirements. On the other hand, reputed multinational consumer goods corporation such as Nestle and Unilever are committed to sourcing CSPO to enhance their reputation and branding as responsible entities in the palm oil industry (Gassler and Spiller, 2018). However, the availability of almost 15 different standards and labels for palm oil creates confusion among consumers and other entities in the palm oil supply chain (Rival *et al.*, 2016).

The requirement for establishing detailed product labels, such as palm oil-free and certified palm oil, and anti-palm oil campaigns to enhance customer awareness and acceptance have distinguished palm oil from other vegetable oils (Hinkes, 2020). These labels can be interpreted as discriminatory since it influences consumer buying patterns by wrongfully indicating that palm oil may be dangerous to human health. Such labels can be deemed an unwarranted reinforcement of the misconception that palm oil is a potential health risk. Consequently, it may ignite a European market inclination to shun palm oil by manipulating consumers' expectations or apprehension.

There exists reasonable justification that the various strategies implemented to limit the usage of palm oil in the trading bloc may be detrimental to the Malaysian palm oil industry. The agricultural commodity is one of the major contributors to the country's Gross Domestic Product (GDP) as the industry exports more than 80% of its domestic production of palm oil and its related products (MPOC, 2021b). As an export-oriented industry, any changes in the importation requirements by consumer countries may disrupt the palm oil supply chain. The stringent requirement for sustainable production, trade and consumption of palm oil for the past several decades implemented by the EU may be extended for consideration by other major importing countries, such as India and China, in the future. Although these two countries

have lower demand for sustainably produced and sourced palm oil currently, global emphasis on sustainable development may motivate the nations to adopt higher sustainability standards when importing agricultural commodities. More so when the EU is often viewed as the trendsetter in benchmarking and standardisation of the global oils and fats industry.

Nutritional benefits of palm oil consumption.

Apart from using labels as a form of discernment, palm oil has also encountered issues regarding its perceived nutritional content and linkage with diseases. The health dimension of palm oil consumption, such as benefits and risks has been prevalent among agri-food actors in the supply chain and consumers for several decades. Research has indicated that palm oil contains triacylglycerol and carotenes that could reduce cholesterol and minimise vitamin A deficiency, respectively (Ruggeri and Samoggia, 2018). In fact, the nutraceuticals and phytonutrients content in palm oil reduces cholesterol and diabetic levels in adults (Naidu and Moorthy, 2021). Notably, there is no scientific proof linking palm oil consumption with higher incidents of cancer, at least for the current time being (Gesteiro *et al.*, 2019). Studies have shown that tocotrienols in palm oil have potential anti-cancer properties that improve the therapeutic treatment of human breast cancer (Kushairi *et al.*, 2018). Moreover, the antioxidant properties of palm oil tocotrienols improves bone health and reduces bone loss for the prevention and treatment of bone-related disease (Radzi *et al.*, 2018). In addition, the anti-inflammatory effect of oil palm phenolics may prevent the formation of neurodegenerative diseases such as Alzheimer's and Parkinson's (Weinberg *et al.*, 2018). There is also insufficient evidence linking dietary palm oil intake with obesity (Kushairi *et al.*, 2019). The findings by these researchers have strengthened the neutral and positive effects of palm oil consumption in the context of non-communicable diseases in humans.

Moreover, since palm oil is *trans* fats-free, it serves as a healthier substitute to partially hydrogenated oils in the food industry in Europe (Hinrichsen, 2016). *Trans* fatty acid present in partially hydrogenated vegetable oil such as soybean oil has proven to have damaging effects on cardiovascular disease and coronary heart disease (Kushairi *et al.*, 2018; Ruggeri and Samoggia, 2018). As a suitable substitute for *trans* fatty acid in food formulations, palm oil is blended with other oil to reduce or remove *trans*-fat to produce *trans*-free solid fat (Berry *et al.*, 2019). The formation of *trans* fatty acids is avoided since the natural semi-solid constituency of palm oil does not require hydrogenation for its application in food products.

However, the risks associated with palm oil consumption are related to its content of saturated fats which could lead to cardiovascular disease (Ruggeri and Samoggia, 2018). More importantly, studies have shown that moderate consumption of palm oil, coupled with varied, balanced and sufficient dietary intake, does not present health risks, particularly the risk of cardiovascular diseases and cancer (Gesteiro *et al.*, 2019; Kushairi *et al.*, 2018).

Recent studies on 3-MCPDE and GE have demonstrated the possible risk of palm oil contaminants to human health (EFSA 2016; Gao *et al.*, 2020; O'Brien, 2008; Sulin *et al.*, 2020). Palm oil has a higher potential to produce these contaminants during the refining process, like almost all other oils and fats, although at generally lower levels (Hinrichsen, 2016). Consequently, NGOs have launched campaigns to remove food items with high levels of those contaminants from supermarkets (Nambiappan *et al.*, 2018). Interestingly, the concerns on public well-being can be assuaged by adopting improved processing techniques that would mitigate the levels of 3-MCPDE and GE in palm oil, albeit with an increase in operational cost (Goggin and Murphy, 2018; Hinrichsen, 2016; Kushairi *et al.*, 2019). In addition to these existing contaminants, mineral oil hydrocarbon (MOH) such as mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH) found in oil palm fruits during processing at mills have carcinogenic and/or genotoxic constituents which can cause harmful consequences in overall health (Ahmad *et al.*, 2019; Sdrigotti *et al.*, 2021). The presence of these contaminants can negatively impact the EU's trust in the quality of Malaysian palm oil if the producers are not able to meet the demands for quality and safety standards.

According to a study by the University of Bologna in Italy, health concerns about the consumption of palm oil are the most frequent tweet on Twitter among manufacturers and retailers with consumers, and vice-versa (Ruggeri and Samoggia, 2018). This communication trend indicates increasing interest and concern among the general public regarding palm oil as edible oil and fat in the food sector. As such, there is a need for further scientific investigation on the potential benefits and risks of palm oil consumption, especially on the possible effects at different levels over a period of time. The outcome of these researches serves as scientific evidence to support the nutritional benefits of palm oil and to address the negative health consequences of consuming palm oil. Together, these findings may invalidate adverse claims regarding the safety and quality of palm oil imported into the European market.

In addition to the environmental and health dimension of sustainability, the palm oil industry

has also created positive societal impacts. As the engine of economic growth for an emerging nation, the palm oil sector has contributed directly to poverty eradication and income generation. Being a labour-intensive sector, direct employment was available in the oil palm estates while indirect employment was generated through provision of supplies and related services. The high labour requirements have made oil palm cultivation an important option of livelihood among the rural poor during the early phases of the industry when wages are low and workforce is abundant (Byerlee *et al.*, 2017). Through land development schemes such as Federal Land Development Authority (FELDA), as many as 112 635 families were provided land to plant oil palm trees to create livelihood (Parid *et al.*, 2013). These income gains have catapulted economic development and promoted social advancements in the country especially in the post-independence years.

Further to employment opportunities at oil palm plantations, the palm oil industry has enabled job openings and positions in other spin-off auxiliary services such as agribusiness, transportation, trading/brokering houses, bulking installations, financial services and other supporting industries. As of 2016, the industry provided direct jobs to more than one million people including 644 522 small farmers (WTO, 2018). Oil palm cultivation also provides a supplementary source of income for farmers growing the plant in multi-crop agriculture (Meijaard and Sheil, 2019). Apart from the additional revenue, new job positions in the palm oil sector will also be created under the National Biomass Strategy 2020 (Mohd Shaharin *et al.*, 2013). By 2020, the strategy created an additional 66 000 employment opportunities, which includes 40 000 jobs under the highly-skilled category generated RM30 billion of new income (Normalisa *et al.*, 2021). The multiplier effect created by the palm oil development has indeed resulted in intense economic activities in related sectors throughout the supply chain. Moreover, the economic snowball effects in the palm oil supply chain have a major impact on the reduction of rural poverty while the shared goals among players in the industry, mainly smallholders, helps to lift social solidarity and integration (Rival and Levang, 2014).

Based on the discussion on sustainability challenges, there are trade-offs between environmental protection and economic development. Developed economies such as the EU are more concerned about the conservation of ecological systems and nature, while income generation through export revenue is the priority of developing nations such as Malaysia. However, win-win solutions are available in the palm oil sector. One possible measure to achieve a mutually beneficial outcome for palm oil-producing countries

and palm oil-consuming countries is by managing the narrative of palm oil development. Effective communication on local conservation efforts and sustainable certification standards can build trust and authenticity among actors in the palm oil supply chain to address issues on sustainability. The involvement of the media and NGOs to disseminate a positive voice on palm oil sustainability could help shape an unbiased public opinion on palm oil consumption.

More importantly, a more objective approach to the issues of palm oil development and consumption would be more appropriate to form a balanced view of the commodity. The measures undertaken by stakeholders such as implementing the MSPO certification scheme and disseminating accurate information on CSPO must be communicated in a transparent and committed manner. Therefore, this article recommends that more studies be conducted on the communication mechanism taken to reduce the negative perception of palm oil in the EU. Related agencies may creatively promote palm oil through social media and other official media communication channels. Bad publicity about palm oil can be effectively countered by providing adequate, timely, and fact-based information.

Although this article provides an overall review of the sustainability challenges in the environmental and health dimension of the palm oil sector, the study is limited to literature accessed from Scopus and Web of Science database only. Hence, this article proposes several suggestions for the consideration of future scholars. Firstly, future review studies related to palm oil sustainability can be conducted using articles from other academic databases such as PubMed and ScienceDirect to diversify the data source. Secondly, there is a need for further research on the plan of action that has been formulated by palm oil-producing countries such as Malaysia. The issues on environmental and health sustainability, as raised in this article, provide the fundamental in understanding the challenges and can be used to study the policy direction in the palm oil industry. Finally, more research using the Systematic Literature Review (SLR) approach are required on various aspects of palm oil sustainability to provide a more complete view of the industry.

CONCLUSION

The article deliberates on the literature on sustainability challenges in the environmental and health dimension of Malaysian palm oil in the EU. Throughout this article, the review demonstrates the importance of sustainable production and consumption of palm oil as a priority to enter the

European market. Although the EU had initially supported the import of palm oil as feedstock for biodiesel production, there has been a change in policy direction over the years. The historical narrative on the evolution of biofuel policies in the EU has shown that the changes mandated by political institutions indicate a diminishing endorsement of palm oil-based biodiesel. Therefore, the reduction in demand for palm oil-based biodiesel can be detrimental to the Malaysian palm oil sector.

Moreover, the EU requirement for CSPO as a validation of traceable and sustainably sourced palm oil further augments the challenges faced by agri-food industry players. The complexity of the international palm oil supply chain, diversity in buyers, numerous industry-guided certification and high substitutability in many end uses of palm oil can impact effective sustainability governance. Nevertheless, the continuous monitoring of food safety and authenticity of palm oil and its products remains one of the main focuses of the industry in ensuring compliance with the latest international food standards and safety regulations. The adherence suggests the continued commitment of the palm oil industry in observing established trading requirements. Amidst fulfilling international safety and quality regulations, there is increasing evidence demonstrating the potential health-promoting effects of nutraceuticals and phytonutrients derived from palm oil. In spite of the findings, it is more important to consider other factors in determining overall well-being such as dietary intake, type and quantity of oil and fats, and lifestyle habits.

With sustainability goals at the global forefront, supporting the sustainable production of palm oil is a constructive approach compared to constraining oil palm cultivation and boycotting the palm oil industry. Although limiting and banning the production, trade and consumption of palm oil appear as a legitimate expression of environmental and health concerns, these actions tend to punish both non-compliant and compliant actors in the palm oil supply chain. More importantly, there is a need to comprehend the risks and consequences of such interventions in the EU and Malaysia, and to what extent these boycotts and restrictions translate into positive actions in the oil palm industry, especially in producing countries. Instead of boycotting palm oil which may reduce the sustainability standards, it is more significant to raise the overall standards and practices on sustainable production. Balanced, ethical and pragmatic perspectives and measures on sustainable development can produce mutual benefits for both producers and consumers in the palm oil industry. As such, the increase in accountable and responsible practices in the palm oil industry should be strategically and consistently

encouraged. This would strengthen the industry ability to address multi-dimensional challenges and allegations as well as remaining sustainable simultaneously.

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In Vitro STUDIES ON PALM PHYTONUTRIENTS: A NUTRIGENOMIC REVIEW

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ABSTRACT

This review discussed the nutrigenomics aspects of palm phytonutrients. Nutrigenomic is the study of the effect of nutrient intake in a specific diet on the regulation of gene expression. Nutrigenomic studies are done to develop personalised dietary, supplements and medicinal products. This can be achieved by either experimenting via in vitro, in vivo or clinical trials. However, researchers prefer to conduct in vitro studies as the data can be obtained faster than other modes of studies, primarily to assess dietary supplement safety. In vitro studies are performed outside of a living organism but in a controlled environment. Phytonutrients are natural chemicals that can be found in plant foods. Palm oil is one of the richest sources of phytonutrients. Palm oil phytonutrients include palm phenolic-enriched fraction (PEF), oil palm phenolics (OPP), and tocotrienol-rich fraction (TRF). These phytonutrients are used as health supplements and were proven to have nutritional benefits for consumers. In this review, we will discuss the nutrigenomics aspects of palm phytonutrients effect on cell proliferation, apoptosis and insulin resistance pathway, based on in vitro data published from 2010.

Keywords: apoptosis, cell lines, gene regulation, insulin resistance, tocotrienol.

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INTRODUCTION

Phytonutrients are bioactive food components that can act as supplements to prevent or/and treat diseases and other physiological disorders. Palm oil is one of the richest sources of phytonutrients, and this oil consists of 1% of phytonutrients, such as carotenes, phytosterols, squalene, ubiquinone, polyphenols, and phospholipids (Loganathan *et al.*, 2010). All of these phytonutrients were reported by many researchers to give nutritional benefits to consumers, both healthy individuals and patients with certain diseases (Loganathan *et al.*, 2010). Palm oil is also a source of vitamin E (Vit E), with its major constituents of 70% tocotrienols (T3) and 30% tocopherols (TCP), and contains 44% palmitic acid (C16:0) and 40% oleic acid (C18:1) (Montoya *et al.*, 2014).

Nutrigenomics is the study of the impact of nutrition intake on genome expression (Elsamanoudy *et al.*, 2016). This genome expression covers transcriptome, proteome and metabolome. According to Munshi and Duvvuri (2008), the research on nutritional genomics will explain the molecular basis on which specific genotypes react to dietary components, thus providing targeted treatment for disease prevention and cure. Nutrients and bioactive food compounds were proven tremendously in publications (Farhud *et al.*, 2010; Fenech *et al.*, 2011; Neeha and Kint, 2013; Sales *et al.*, 2014; Walker and Blackburn, 2004) that these two components can modify cellular pathways through gene expression. The identification of these gene-nutrient interactions will facilitate the recommendation of genotype-specific diets (Sales *et al.* 2014). For example, those people who are lactose intolerant could not break down the main milk sugar, known as lactose, effectively in dairy products. This group of people are advised to limit foods containing lactose or to consume lactase supplements or lactose-free dairy products (Swallow, 2003).

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Many publications discussed the nutritional effects of consuming palm oil and its phytonutrients. Sundram *et al.* (2003) discussed several effects of the bioactive compounds in the phytochemistry of palm oil fruit on its nutritional and physiological benefits, such as its antioxidant activities, ability to lower cholesterol, anti-cancer effects and ability to prevent atherosclerosis. Balasundram *et al.* (2005) also discussed the antioxidant properties of palm fruit extracts. In 2014, May and Nesaretnam discussed the effects of palm oil on blood lipids and lipoproteins. Dong *et al.* (2017) published an article on the effect of red palm oil on vitamin A deficiency. There are also a few publications that focused on the phytonutrients themselves, such as the effects of tocotrienol-rich fraction (TRF) (Ali and Woodman, 2015; Mazlan *et al.*, 2017; Meganathan and Fu, 2016) and also the effects on oil palm phenolics (OPP) consumption (Che Idris *et al.*, 2014; Patten *et al.*, 2015).

Even though there are many publications discussed on the nutritional benefits of consuming palm oil and its phytonutrients, most of these publications only focused either on its nutritional properties or one of its phytonutrients. None of them focused on how the experiments were conducted. Therefore, we believed that there is a need to discuss on how the experiments were conducted, to give an overview and guidance to other researchers for their future studies. There are two types of experiments conducted by researchers; *in vitro* and *in vivo*. Both are Latin words, in which *in vitro* means “within the glass”, and *in vivo* means “within the living”. *In vitro* studies are defined as experiments that are carried out in a controlled environment, outside of a living organism. *In vivo* studies are studies that are done on a living organism, such as plant and animal. *In vitro* studies are usually conducted using specific human or animal cells, bacteria and viruses. It is cheaper,

easier and safer as compared to other studies. Even though the data obtained from *in vitro* studies give some insights and predictions on certain aspects, not all *in vitro* data reflects the living organism. Nevertheless, *in vitro* studies are preferable to assess dietary supplement safety as data could be obtained faster than *in vivo* studies.

Hence, this review aims to provide an integrated assessment of the available *in vitro* data on the nutritional genomic aspects of palm phytonutrients effect, based on studies published from 2010 onwards.

NUTRIGENOMIC EFFECTS ON CELL PROLIFERATION

A cell cycle is a process where cells replicate and make two new cells. The cell cycle has two stages: The interphase and the mitotic phase (M phase). The interphase consists of the Gap 1 (G_1) phase, the synthesis (S) phase and the Gap 2 (G_2) phase, while the M phase is where the division of the nucleus and cytoplasm occurs (Alberts *et al.*, 2002) (Figure 1). However, cells may enter a specialised resting state, known as Gap 0 (G_0) prior to G_1 , if the extracellular environments are unfavourable. The cell's progression is regulated by a family of proteins known as the cyclin by activating the cyclin-dependent kinase (CDK) enzymes (Galderisi *et al.*, 2003). Srivastava and Gupta (2006) demonstrated that chemotherapeutic and chemopreventive drugs can have antiproliferative effects by causing cell division to stop at specific cell cycle checkpoints.

In 2010, Kunnumakkara *et al.* (2010) used four types of pancreatic cell lines: BxPC-3, MIA PaCa-2, Panc-1 and MPanc-96; and discovered that gamma-tocotrienol (γ -T3) inhibited all of these four pancreatic cell lines from proliferating. They found that the inhibition was in a dose- and time-

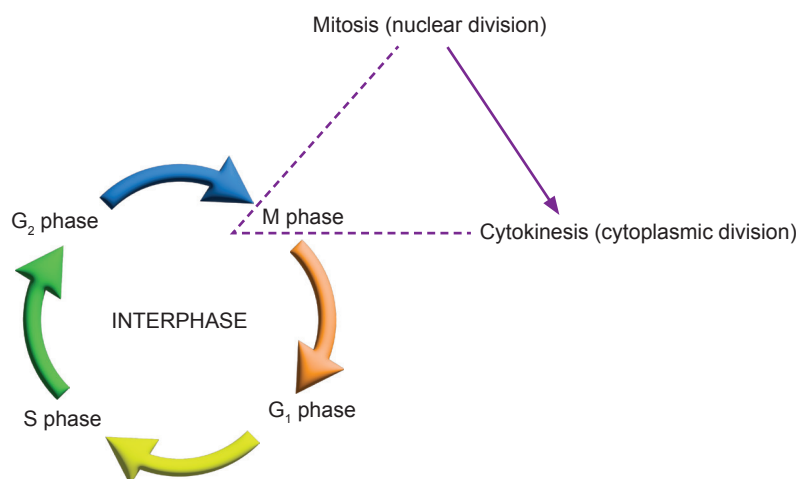


Figure 1. The cell cycle, comprising of four phases: G_1 phase (growth), S phase (DNA replication), G_2 phase (growth and preparation for mitosis) and M phase (cell division).

dependent manner, in which they compared the proliferation rate using two concentrations of γ -T3; 10 mM and 50 mM; for 2, 4 and 6 days. The cell proliferation was almost entirely stopped at the concentration of 50 mM. The inhibition was because of the cell cycle arrest at the G_1 phase. In addition, they discovered that the inhibition of cell proliferation by γ -T3 might be explained by the downregulation of cell proliferative gene products, such as *cyclin D1*, *c-Myc* and *COX-2* (Kunnumakkara *et al.*, 2010).

Wu and Ng (2010) treated human cervical carcinoma (HeLa) cells with three types of T3 (α -T3, γ -T3 and δ -T3) and a-TCPs discovered at the concentrations of 1, 2 and 3 mM, both α -T3 and γ -T3 significantly caused cell cycle arrest at G_0/G_1 phase. However, they found that the effects of T3 on the distribution of the cell cycles depend on the cell cycle states. This is because α -T3 downregulated the expression of cyclin D3, p16 and CDK6 but does not have any effect on cyclin D1, P15, P21, P27 and CDK6. Contrarily, γ -T3 does not affect all these proteins.

Luna *et al.* (2011) used human intestinal fibroblast (HIF) isolated from Crohn's disease (CD) patients in their experiment. They treated the cell with 1 mM of TRF and discovered that this concentration does not have any effects on the HIF proliferation. However, when they increased the concentration of TRF doses between 10 to 1000 mM for 24 hr, they found that these concentrations reduced the HIF proliferation with plateau effects starting at 20 mM of TRF. Unfortunately, they did not further investigate to determine the phase in the cell cycle that was affected by the TRF treatment.

Lim *et al.* (2014) used two types of cell lines: human lung adenocarcinoma (A549) and human glioblastoma grade IV (U87MG), to test the different concentrations of palm oil derived β -T3. They tested the concentration of 2 and 10 mM on the A549 cell line and 3 and 60 mM on the U87MG cell line at three timepoints: 24, 48 and 72 hr. They found that these concentrations impressively reduced the cell viability in a concentration- and time-dependent manner. They found that the decrease in cell numbers and increase in cell death only happened in cancerous cell lines but not in non-cancerous cells. They also revealed that β -T3 significantly increased the A549 and U87MG cells arrested at the G_0/G_1 stage at 24 and 48 hr.

Rati Selvaraju *et al.* (2014a) demonstrated that both TRF and a-TCP were able to protect and repair cell injury. They exposed the human neuroblastoma cells (SK-N-SH) to 120 mM glutamate to induce injury in the cells. When treating these cells with TRF and a-TCP, they discovered that the concentration of 100 to 300 ng/mL of TRF and 200 ng/mL of a-TCP significantly increased the cell proliferation in the G^1 and S phases of the cell cycle

in the cell line. Cells were not significantly changed in the G^2/M phase after the treatment of TRF/a-TCP.

Concurrently, the same research group carried out the same treatment using human glioblastoma cells (DBTRG-05MG) to observe the protective effects of TRF and a-TCP against glutamate injury (Rati Selvaraju *et al.*, 2014b). They injured the cells by using 180 mM glutamate. Their data showed that cell numbers increased with treatment of 100, 200 and 300 ng/mL of TRF and a-TCP, in the S phase.

In 2015, Ye *et al.* demonstrated the anti-cancer effect of δ -T3 on two different types of bladder cancer cell lines, which were the T24 and 5637 cell lines. The cell lines were treated with various concentrations of δ -T3, starting from 0, 50, 100 and 150 mM for 48 hr. Their analysis using flow cytometry revealed that treatment with δ -T3 of 100 mM and more, resulted in G_1 phase arrest and a decrease in the number of cells in the S phase. They further investigated the amounts of protein expression for the cell cycle and discovered the elevated expression levels of cell cycle inhibitors, p21^{Waf1/Cip1} and p27^{Kip1}, and decreased expression levels of Cyclin D1 after 24 hr of treatment with δ -T3 at all dosages in T24 and 5637 cells (Ye *et al.*, 2015).

Ji *et al.* (2015) revealed that OPP was able to promote cell cycle arrest in the S phase for two types of pancreatic cancer cell lines: PANC-1 and BxPC-3; in a dose-dependent manner. For the PANC-1 cell line, the treatment group with 40 mL/mL of OPP, had roughly 28% of the cells in the S phase compared to 25% in the non-treated cells. The same pattern was shown in the BxPC-3 cell line treated with 50 mL/mL of OPP, where 52% of cells were found in the S phase, compared to the non-treated cells, with 21%.

NUTRIGENOMIC EFFECTS ON APOPTOSIS

Apoptosis is programmed cell death. It was first described back in 1972 (Kerr *et al.*, 1972). Apoptosis is a homeostatic process that happens to maintain cell populations in tissues. It also occurs as a defence mechanism in the immune response or when cells are exposed to a disease or dangerous chemical reagents (Norbury and Hickson, 2001). During apoptosis, the cells shrink, with increased cytoplasm density, followed by organelles clumping with each other. These changes in cellular morphology are observable via light microscopy. Apoptosis involves the activation of caspases, a group of cysteine proteases and a cascade of events related to actuate stimuli to the final demise of the cell (Elmore, 2007). Caspases' function is to inactivate proteins that are important to cell survival (Kannan and Jain, 2000). There are two types of caspases activation pathways; extrinsic pathway and intrinsic

pathway. The extrinsic pathway is regulated by tumor necrosis factor (TNF) receptor, while the intrinsic pathway occurs in the presence of cellular stresses that involve cell organelles. *Figure 2* shows the schematic representation of apoptotic events.

Wu and Ng (2010) showed that α -T3 and γ -T3 greatly induced apoptosis in the HeLa cell line, as the concentration of these both compounds increased. They started with 0 mM of α -T3 and γ -T3, followed by 1, 2 and 3 mM. The HeLa cells were stained with annexin V-FITC and they found that the proportion of stained cells increased in both α -T3- and γ -T3-treated cells, through the fluorescence activated cell sorting (FACS) analysis. The presence of phosphatidylserine on the cell surface after 24 hr of treatment, proved that this both compounds triggered cell apoptosis.

Luna *et al.* (2011) also showed that TRF-induced apoptosis in a caspase-dependent process. They treated two HIF cell lines derived from CD patients and ulcerative colitis (UC) patients with 20 mM TRF. After 24 hr, they discovered an increase in the caspase-8, caspase-9 and caspase-3 activity. In addition, they also found that the pan-caspase inhibitor, zVAD-fmk, completely abolished HIF apoptosis, thus proving that the apoptosis of HIF caused by TRF is a caspase-dependent process.

In another study done by Lim *et al.* (2014), they treated 10 mM of β -T3 on the A549 cell line and 60 mM of β -T3 on the U87MG cell line to detect caspase-8 initiation. Cells were also preincubated for 30 min with 10 mM and 30 mM of the caspase-8 inhibitor z-IETD-fmk (Z-Ile-Glu-Thr-Asp-fmk) to

determine whether caspase-8 was indeed involved. Both A549 and U87MG cell lines were shown to induce caspase-8 activity in response to β -T3. When compared to A549 cells, β -T3 consistently caused more caspase-8 activation in U87MG. An obvious reduction in β -T3 induced caspase-8 activity was also shown in the presence of z-IETD-fmk, which was somewhat linearly proportional to the concentration of z-IETD-fmk used (Lim *et al.*, 2014).

In 2015, researchers discovered that phenolic-enriched fraction (PEF) enhanced cell survival by regulating the genes involved with oxidative stress, which leads to apoptosis (Oskoueian *et al.*, 2015). They used aflatoxin B1 (AFB1)-treated chicken hepatocytes cells. In an untreated cell state, upregulation of pro-inflammatory mediators caused an imbalance between the anti-apoptosis gene, which leads to apoptosis. The genes were *bcl2*, *bax*, and caspase-3 effector. When the cells were treated with gradual concentrations of PEF from 0 to 5, 10, 20 and 40 mg/mL for 24 hr, the *bcl2* gene was increased. However, the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-kB* genes), the pro-inflammatory mediators; nitric oxide synthase (*iNOS*), tumour necrosis factor alpha (*TNF- α*), interleukin-1 beta (*IL1 β*) and interleukin-6 (*IL6*); and *bax* gene, were found to be downregulated. This data showed that PEF regulated the imbalance between anti-apoptotic effectors, which results in cell survival enhancement. *Figure 3* illustrates the cell survival events due to the PEF treatment.

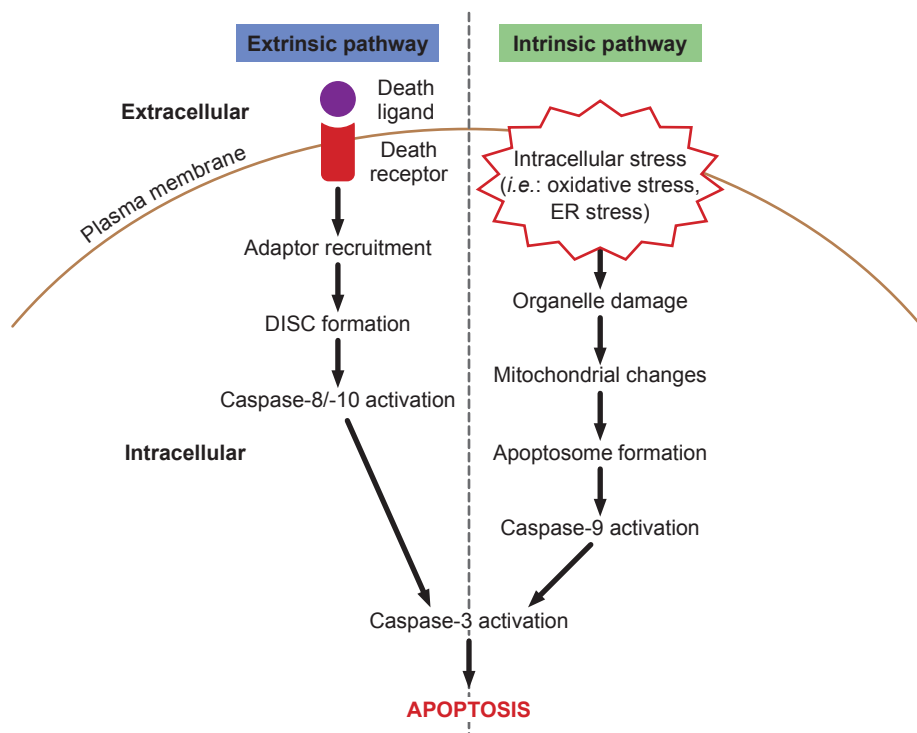


Figure 2. Comparison between the extrinsic and the intrinsic pathway, which leads to apoptosis.

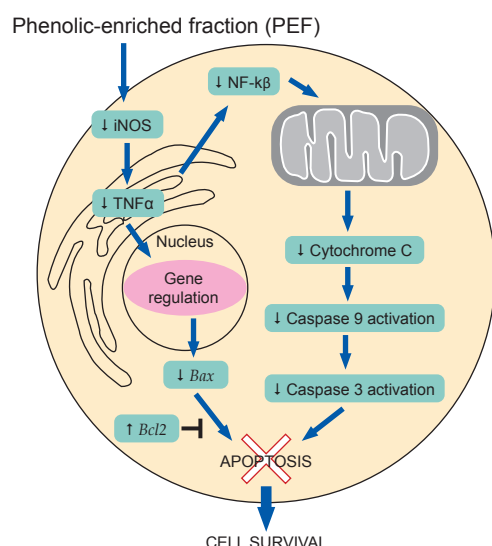


Figure 3. The cell survival events due to the PEF treatment on the AFB1-treated chicken hepatocytes.

In addition, the PEF treatment was shown to upregulate the nuclear factor (erythroid-derived 2)-like 2 (nrf2) protein, while significantly down-regulated the expression of heat shock protein 70 (Hsp70) and caspase-3 proteins. In 2011, nrf2 was reported to activate cellular antioxidant defence mechanisms and enhanced the production of antioxidant enzymes and heat shock proteins (El Golli-Bennour and Bacha, 2011). Hsp70 acts as a sensor of cellular redox exchanges, which act as antioxidant enzymes. Under oxidative stress, this protein can restore and maintain the redox homeostasis of cells.

Contrarily, Ye *et al.* (2015) revealed that the Bax's expression level was increased in all four bladder cancer cell lines that were used: T24, 5637, J82 and UMUC-3, with the treatment of δ -T3. The expression level started to increase as the δ -T3 concentration increased from 0 to 50, 100 and 150 mM. However, the expression levels of Bcl-2, Bcl-xL and Mcl-1's gradually decreased as the increase in the δ -T3 concentration in all four bladder cancer cell lines that were used. The treatment of δ -T3 for 24 hr on these cell lines resulted in the activation of Caspase-3 and cleavage of Poly (ADP-ribose) polymerase (PARP).

Ji *et al.* (2015) used human pancreatic cell lines, PANC-1 and BxPC-3, to study the potential effect of OPP as an anti-carcinogenic agent. Their findings correlate with the apoptotic pathway. Oil palm phenolics are the by-product of oil palm, recovered from aqueous waste in the milling process, containing various water-soluble phenolic compounds. Oil palm phenolics were shown to have valuable medicinal properties such as cardioprotective (Che Idris *et al.*, 2014) and neuroprotective effects (Leow *et al.*, 2013).

According to Ji *et al.* (2015), OPP reduced the production of anti-apoptotic genes, Bcl-XL, and

survivin, in both cell lines gradually, as the OPP concentration increased from 0, 20, 30 to 40 mL/mL, post 72 hr treatment. On the other hand, OPP increased the expression of pro-apoptotic genes gradually, such as caspase-3, caspase-9, and PARP as the same concentration of OPP gradually increased after 72 hr treatment. This illustrates that OPP activates the apoptosis in the cancer cell lines by inducing the pro-apoptotic genes and inhibiting the cell survival genes; thus, OPP can slow down the migration and metastasis process in PANC-1 and BxPC-3 cell lines. An illustration of the apoptosis events is shown in Figure 4.

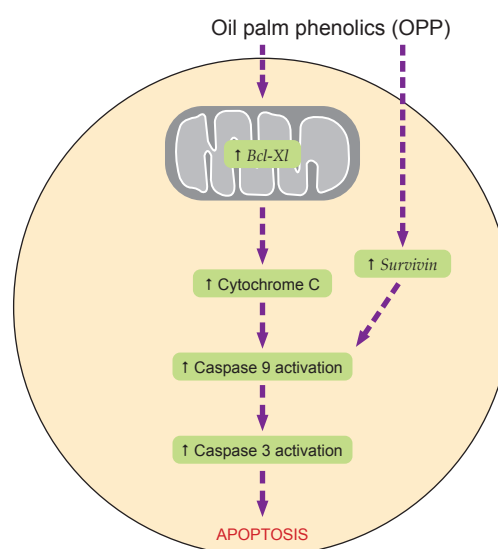


Figure 4. An illustration showing how oil palm phenolics (OPP) initiates apoptosis to slow down the metastasis process on human pancreatic cell lines.

On another note, endoplasmic reticulum (ER) stress (EndoR stress) is an intrinsic pathway that leads to apoptosis. Endoplasmic reticulum was proposed to modulate apoptosis by sensitising mitochondria to various extrinsic and intrinsic death stimuli and commencing their own cell death signals. Endoplasmic reticulum stress is influenced by pharmacological agents that either hinder N-linked glycosylation, obstruct ER to Golgi transport, damage disulfide bond formation, or hamper ER Ca^{2+} stores. There are three ER transmembrane receptors; pancreatic ER kinase (PERK)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) (Li *et al.*, 2014). In normal conditions (non-stressed cells), all of these three sensors are in an inactive state through binding to the ER chaperone BiP. However, once ER is stressed, excessive unfolded proteins will gather in the ER lumen, causing BiP to dissociate from the ER stress transducer. This initiates the activation of unfolded protein response (UPR) and promotes cell survival (Bertolotti *et al.*, 2000; Li *et al.*, 2014; Zhang

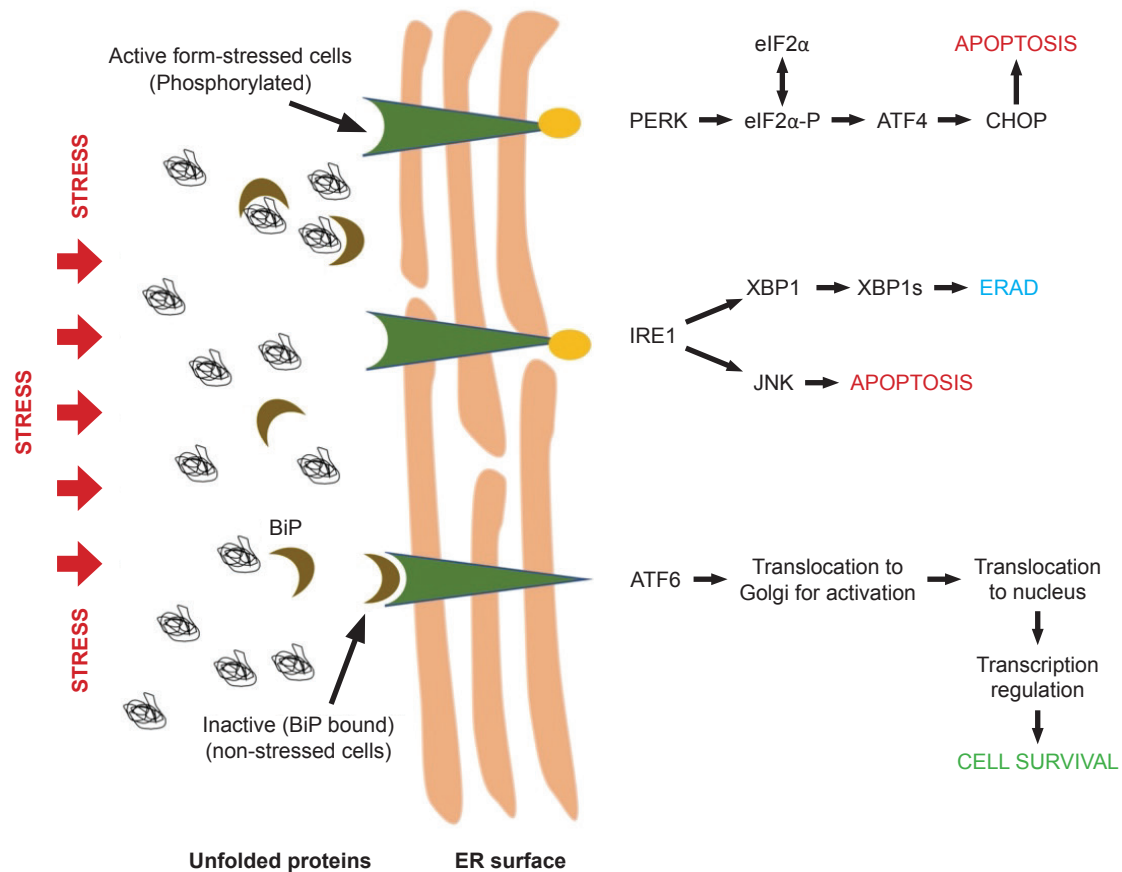


Figure 5. The endoplasmic reticulum (ER) signalling pathways.

et al., 2010) (Figure 5). It was reported that apoptotic response occurs if the damage is too substantial and homeostasis could not be restored within a certain period (Breckenridge *et al.*, 2003; Patil and Walter 2001). Pancreatic ER kinase-like ER kinase has both pro-and anti-apoptotic functions, while ATF6 is anti-apoptotic and IRE1 is pro-apoptotic (Szegezdi *et al.*, 2006).

In a journal published by Comitato *et al.* (2016), they used HeLa cells to investigate the effect of T3 in gene expression that leads to the activation of EndoR stress and Ca-dependent. They demonstrated that γ - and δ -T3 activated a series of specific cellular responses leading to apoptosis. Among the differentially expressed genes during these treatments, seven genes were reported to function as markers of EndoR stress. Their data showed that at a concentration of 10 mg/mL, both γ -T3 and δ -T3 were able to increase the expression of heat-shock 70 kDa protein 5 (HSPA5) and asparagine synthetase (ASNS) after 24 hr. Heat-shock 70 kDa protein 5 was suggested to promote protein folding and inhibits protein aggregation in EndoR (Meunier *et al.*, 2002), while ASNS is a transcriptional target of ATF4 in response to amino acid starvation through the GCN2/eIF2 α axis (Zhang *et al.*, 2014).

Treatment with 10 mg/mL of γ -T3 specifically was shown to increase the gene expression of TNF receptor superfamily member 6 (Fas), x-box binding protein 1 (XBP-1), and C/EBP homologous protein (CHOP). Figure 6 shows the illustration of the apoptosis events due to the T3 treatment on the HeLa cells (Comitato *et al.*, 2016).

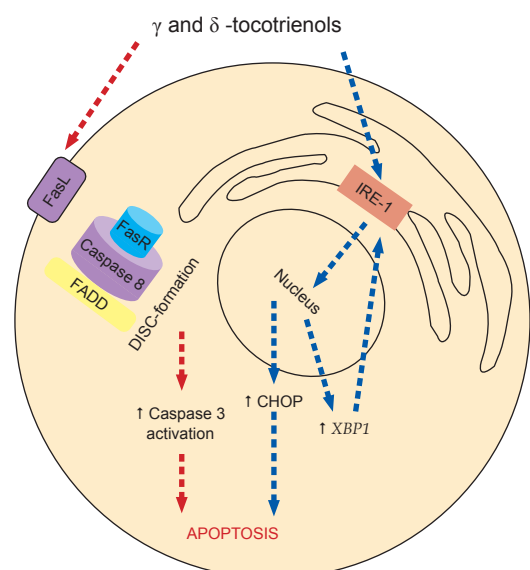


Figure 6. The effects of γ - and δ -tocotrienols treatments on HeLa cell line.

Fas is a death receptor family, also known by other names such as APO-1 and CD95 (Krammer, 2000). *Fas* expression will lead to the formation of the CD95 death-inducing signalling complex (DISC), which will eventually lead to either cell survival or cell death, depending on the downstream mechanism of DISC (Lavrik and Krammer, 2012). X-box binding protein 1 was reported to bind promoters of several genes involved in the UPR pathway and ERAD (ER-assisted degradation) to restore protein homeostasis and promote cytoprotection (Sano and Reed, 2013). Activation of CHOP cause changes in gene expression that favour apoptosis (such as the *BCL2* gene).

In addition, treatment with only δ -T3 increased the expression of tribbles pseudokinase 3 (*TRIB3*) and stromal cell-derived factor 2-like 1 (*SDF2L1*). *Tribbles pseudokinase 3* is one of the pro-apoptotic genes regulated when CHOP is overexpressed (Zhang and Kaufman, 2008). Stromal cell-derived factor 2-like 1 was reported to be involved in the regulation of caspases, which leads to apoptosis (Lorenzon-Ojea *et al.*, 2016). Thus, Comitato *et al.* (2016) concluded that both treatments of γ -T3 and δ -T3 could affect UPR.

Studies that showed palm phytonutrients' effect on apoptosis suggest that palm phytonutrients may act as an anti-tumour agent. In 2019, TRF was shown to positively affect the anti-tumour immune response (Abdul Hafid and Radhakrishnan, 2019). Bone marrow-derived dendritic cells were used. Tumour lysate from 4T1 murine mammary cancer cells was added to the dendritic cells. Researchers treated these cells with five types of treatment, with one treatment was given 8 mg/mL of TRF for 24 hr. The TRF treatment was able to promote the survival of T-cells in the tumours. It showed the slowest tumour growth and increased the rate of cell survival.

Four genes were differentially expressed, which were cysteine-rich protein 2 (*CRIP2*), interferon-induced transmembrane protein 1 (*IFITM1*), mannose receptor C type 1 (*MRC1*), and special AT-rich binding protein 1 (*SATB1*). Interferon-induced transmembrane protein 1 (*IFITM1*), *MRC1*, and *SATB1* were found to be down-regulated. However, at a confidence level of 99%, only *SATB1* was reported to be significantly down-regulated. Studies have shown that the function of *SATB1* is to fasten the specialised DNA sequences and bind to the matrix attachment region (MAR) (Kohwi-Shigematsu *et al.*, 1997), which is involved in the loop domain organisation of chromatin remodelling. Special AT-rich binding protein 1 (*SATB1*) was also found to be engaged with prognostic outcomes of cancer patients (Alvarez *et al.*, 2000). Thus, they concluded that TRF treatment could delay tumour growth, and *SATB1* could be the next potential target gene in anti-tumour studies.

NUTRIGENOMIC EFFECTS ON INSULIN RESISTANCE PATHWAY

Insulin resistance is usually associated with obesity. Insulin promotes glucose uptake via the activation of the phosphatidylinositol 3-kinase pathway, which is responsible for most of the metabolic actions of insulin. Many genes are associated with insulin resistance. Among them is peroxisome proliferator activated receptor gamma (*PPARG*), prospero homeobox protein 1 (*PROX1*), insulin-like growth factor 1 (*IGF-1*), insulin receptor substrate 1 (*IRS1*), fat mass and obesity-associated (*FTO*) and many more.

In a journal published by Zaulkffali *et al.* (2019), the neuroblastoma cell line, SK-N-SH, was used to analyse the influence of vitamin (vit) D and E in enhancing insulin resistance in the neuronal-insulin resistance model. The Vit E was in the form of TRF. Treatment with 200 ng/mL Vit E alone and with the addition of 10 ng/mL and 20 ng/mL Vit D did not elevate the expression level of glucose transporter 3 (*GLUT3*) and glucose transporter 4 (*GLUT4*). These are insulin signalling markers. Glucose transporter 3 (*GLUT3*) is the main glucose transporter in the brain. Vitamin E alone was able to increase 2-D-glucose uptake significantly. In addition, treatment with the same concentration of Vit E alone and the combination of Vit E and D were shown to reduce the expression level of glycogen synthase kinase 3 beta (*GSK3 β*) by almost 2-fold substantially. Vitamin E was found to also significantly reduce *TAU* expression level by 1-fold compared to the negative control. Vitamin E in the form of TRF was found to significantly downregulate reactive oxygen species (ROS) production after the induction of chronic insulin resistance, even though it did not substantially affect the insulin signalling molecules. Figure 7 illustrates the pathway of the insulin resistance model.

In another study, the *Adfp* (adipophilin) gene was expressed in human skeletal muscle (Phillips *et al.*, 2005). Adipophilin was previously reported to stimulate the uptake of fatty acids (Gao and Serrero, 1999) and increased the storage of triacylglycerol (TAG) (Listenberger *et al.*, 2007). Adipophilin was highly expressed when treated with palmitic acid and oleic acid, using a C2C12 cell line (De Wilde *et al.*, 2010). The C2C12 cell line is a mouse muscle myoblast cell. This gene was expressed more in the oleic acid treatment as compared to palmitic acid. The expression of the *Adfp* gene is regulated by the peroxisome proliferator-activated receptors (PPAR) family. All three subtypes of PPAR; PPAR α , PPAR γ , and PPAR β/δ ; increase the *Adfp* gene expression in a specific way. However, this gene was expressed the most through the activation of PPAR α . Activation of PPAR α reduces TAG level; thus, this correlates with the earlier findings in 1999 and 2007 (Gao and Serrero, 1999; Listenberger *et al.*, 2007).

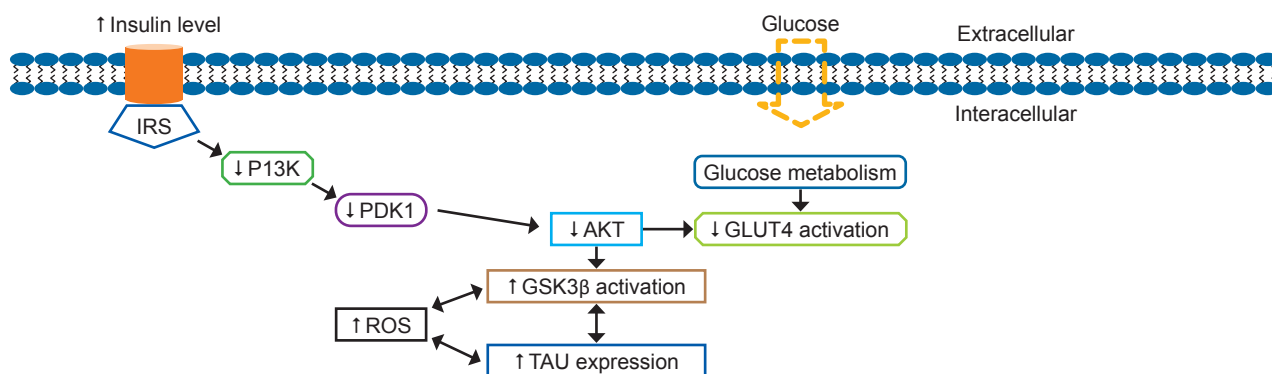


Figure 7. Insulin resistance model pathway.

TABLE 1. SUMMARY OF *In vitro* STUDIES ON THE NUTRIGENOMICS EFFECTS OF PALM PHYTONUTRIENTS

Author(s)	Year published	Cell line(s)	Types of palm phytonutrient(s)	Nutrigenomic effect(s)
Kunnumakkara <i>et al.</i>	2010	Pancreatic cell lines (BxPC-3, MIA PaCa-2, Panc-1 and MPanc-96).	γ-T3	Effects on cell proliferation.
Wu and Ng	2010	Human cervical carcinoma cell line (HeLa).	a-T3, g-T3, d-T3 and a-TCPs	Effects on cell proliferation and apoptosis.
Luna <i>et al.</i>	2011	Human intestinal fibroblast (HIF) isolated from Crohn's disease and ulcerative colitis patients.	TRF	Effects on cell proliferation and apoptosis.
Lim <i>et al.</i>	2014	Human lung adenocarcinoma (A549) and human glioblastoma grade IV (U87MG) cell lines.	b-T3	Effects on cell proliferation and apoptosis.
Rati Selvaraju <i>et al.</i>	2014a	Human neuroblastoma cell line (SK-N-SH).	TRF and a-TCP	Effects on cell proliferation.
Rati Selvaraju <i>et al.</i>	2014b	Human glioblastoma cell line (DBTRG-05MG).	TRF and a-TCP	Effects on cell proliferation.
Ye <i>et al.</i>	2015	Bladder cancer cell lines (T24, 5637, J82 and UMUC-3).	d-T3	Effects on cell proliferation and apoptosis.
Ji <i>et al.</i>	2015	Pancreatic cancer cell lines (PANC-1 and BxPC-3).	OPP	Effects on cell proliferation and apoptosis.
Oskoueian <i>et al.</i>	2015	Aflatoxin B1 (AFB1)-treated chicken hepatocytes cell.	PEF	Effects on apoptosis.
Comitato <i>et al.</i>	2016	Human cervical carcinoma cell line (HeLa).	γ- and δ-T3	Effects on apoptosis.
Abdul Hafid and Radhakrishnan	2019	4T1 murine mammary cancer cell.	TRF	Effects on apoptosis.
Zaulkffali <i>et al.</i>	2019	SK-N-SH	TRF	Effects on insulin resistance pathway.

CONCLUSION

In vitro studies may lead to results that do not complement the situation of a living organism. However, this type of study allows a substance to study safely without compromising the health safety of animals and humans. Preliminary data obtained from here will be evaluated, and only those that appear to be beneficial will proceed to animal or human studies. The above review discussed the effects of palm phytonutrients on genetic modulation through *in vitro* experiments. Table 1 summarised the findings that were discussed from the year 2010 onwards.

The benefits of consuming palm oil and its phytonutrients have been widely discussed and published in journals, articles, or even through conferences and lectures. These benefits go beyond nutritional aspects, where many *in vitro* studies

have reported findings indicating the potential for medicinal applications. We believed that the *in vitro* research that was published in all these previous publications could help and guide scientists to propose new projects that will be done *in vivo* and to investigate deeper in clinical research.

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AN ASSESSMENT OF THE MALAYSIAN OIL PALM BREEDING POPULATIONS USING AFLP MARKERS

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ABSTRACT

The narrow genetic diversity coupled with intensive selection has reduced the variability of yield components and vegetative traits of oil palm breeding populations. Thus, the objective of the current study is to evaluate the genetic variability of oil palm breeding populations using amplified fragment length polymorphism (AFLP) markers. The eight AFLP primer pairs utilised in this study generated 228 bands across 67 populations. As expected, populations created from intercrossing revealed relatively higher levels of genetic diversity compared with those derived from selfings. The dendrogram and Principal Component Analysis (PCA) of the DxP palms indicated a high genetic similarity. Among palms from the TxT/P crosses, the groupings were aligned according to the agency, signifying the accumulation of distinctive sets of alleles, likely due to the different selection pressure imposed by the respective agency. In PCA, the TxT palms, however, revealed a close genetic relationship indicating the need for incorporating new genetic resources to widen the genetic base. Based on the results, it is recommended the introduction of new genetic resources into the *dura* and *tenera*/*pisifera* populations. Additionally, crossing palms from populations of high genetic distances and adopting intercrossing scheme should result in off-springs with considerable diversity for selection gain in future breeding programmes.

Keywords: AFLPs, breeding populations, genetic assessment, oil palm.

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INTRODUCTION

Selection in plant breeding is aimed at achieving uniformity and improvement of desirable traits of a cultivated variety. However, selection may also lead to the narrowing of genetic diversity and elimination of allelic variants, which can affect crops' ability to withstand any future environmental challenges (Bhandari *et al.*, 2017; Govindaraj *et al.*, 2015). Although uniformity in high yield, quality, growth and maturity are necessary for advanced farming technology, genetically uniform crops

may hold a hidden danger. A classic example demonstrating the vulnerability of a uniform crop is the Corn Belt hybrid that was wiped out due to the devastating disease, southern corn leaf blight (SCLB) caused by the fungus, *Helminthosporium maydis* T. (Ullstrup, 1972). History has further revealed that genetic diversity among cultivated varieties and breeding populations is necessary for sustainability and selection gains.

In oil palm, the cultivated variety *dura* × *pisifera* (DxP) or the resultant *tenera* is produced by hybridising selected *dura* (female) and *pisifera* (male). The *dura* and *pisifera* parental stocks are maintained and improved separately in recurrent selections. DxP progeny testing is carried out to evaluate agronomic performances of various parental combinations (Soh, 1999). The *dura* lines used in commercial DxP seed production in Malaysia are mainly descendants of four palms planted at the Bogor Botanical Garden, Indonesia in 1848 (Rosenquist, 1986). Kushairi (1992) has reviewed the oil palm breeding programmes in

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Malaysia. From Bogor, oil palm seeds reached the “Public Garden” in Kuala Lumpur in 1905, and planted in “Experimental Plantation KL”, which subsequently became the source of *dura* seeds for the Serdang Avenue (SA) palms. Through an alternative route, seeds from Ulu Remis were brought into Malaysia and laid down at Genetic Blocks I to V and VII in Chemara. These SA and the Ulu Remis materials were later intercrossed and planted at Genetic Block (GB) IIIA, Chemara, Layang-layang. Some of the introgressed populations were established at Kelanang Bharu, Banting and used in breeding programmes.

Unlike the *duras*, the genetic source of *pisifera* breeding populations is slightly more diverse. In Malaysia, oil palm breeders utilised *pisifera* populations from Congo, Nigeria and Ivory Coast, among others (Yong, 1992). Two well-known *pisifera* populations from Congo are Yangambi and AVROS. Yangambi lines were developed by National Institute for Agronomic Study of the Belgian Congo (INEAC) in Eala and nine other *teneras* from Yawenda, N’gazi and Isangi (Hardon *et al.*, 1976). A descendant of the Yangambi palm, SP540 was developed in Sungei Panchur, Indonesia. The SP540 palm was crossed to an African *tenera* by *Algemeene Vereniging van Rubberplanters ter Oostkust van Sumatra* (AVROS) (Rosenquist, 1986), where *pisifera* descendants of the crosses were simply called AVROS *pisifera* and also used in breeding and evolved into Ulu Remis *tenera* (URT) (Rosenquist, 1986).

In oil palm, breeding and selection had reduced the coefficient of variation (CV) for fruit bunch yield (Kushairi, 1992; Kushairi *et al.*, 1994; Lee *et al.*, 1990) and decreased the variability in terms of fresh fruit bunch (FFB) components and vegetative traits among some DxP progenies (Noh *et al.*, 2010). Diversity of an intended crop can be investigated by both, phenotypic traits and DNA-based methods such as Restriction Fragment Length Polymorphism (RFLP),

Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), microsatellites and Single Nucleotide Polymorphism (SNP) (Bhandari *et al.*, 2017). AFLP has been applied for genetic diversity studies in a wide range of plant species including teak (Vaishnaw *et al.*, 2015), orchardgrass (Zhang *et al.*, 2018), potato (Bryan *et al.*, 2017), legume shrub (Fan *et al.*, 2017), winged bean (Mohanty *et al.*, 2019), blackberry (Garrido *et al.*, 2020), *Jatropha* (Avendaño *et al.*, 2015) and *Brassica oleracea* (El-Esawi *et al.*, 2016). The main advantage of AFLP markers is that several loci can be assayed simultaneously, where the technique is amenable to automation and more importantly shows high stability and reproducibility (Thaipong *et al.*, 2017; Todd *et al.*, 2011).

In this study, the genetic variability of 67 oil palm breeding populations in Malaysia was estimated by employing eight AFLP primer combinations. The information generated could help breeders to plan and strategise crossing schemes to prevent inbreeding and maintain sufficient variability among breeding populations for selection gain in future breeding programmes.

MATERIALS AND METHODS

Planting Materials

A total of 67 oil palm breeding populations were sampled from six Malaysian oil palm research organisations (Table 1). Of the 67 populations, 39 were Dx/D, 10 Dx/P and 18 Tx/T/P populations. The Dx/D and Tx/T/P populations were derived from 10 and six different genetic backgrounds respectively. Between eight and 10 palms were sampled for each population. DNA was extracted from leaf materials sampled from each palm using a modified CTAB method (Rahimah *et al.*, 2006).

TABLE 1. LIST OF Dx/D, Dx/P, Tx/T/P POPULATIONS INCLUDED IN THE STUDY

No.	Population name	Crossing scheme	Agency	Cross type
1	AG1D1	self	Agency 1	DxD
2	AG1D2	intercross	Agency 1	DxD
3	AG1D3	intercross	Agency 1	DxD
4	AG1D4	intercross	Agency 1	DxD
5	AG2D1	self	Agency 2	DxD
6	AG2D2	intercross	Agency 2	DxD
7	AG2D3	intercross	Agency 2	DxD
8	AG2D4	intercross	Agency 2	DxD
9	AG2D5	self	Agency 2	DxD
10	AG2D6	self	Agency 2	DxD

TABLE 1. LIST OF DxD, DxP, TxT/P POPULATIONS INCLUDED IN THE STUDY (continued)

No.	Population name	Crossing scheme	Agency	Cross type
11	AG2D7	intercross	Agency 2	DxD
12	AG2D8	intercross	Agency 2	DxD
13	AG2D9	intercross	Agency 2	DxD
14	AG2D10	intercross	Agency 2	DxD
15	AG2D11	intercross	Agency 2	DxD
16	AG3D1	self	Agency 3	DxD
17	AG3D2	self	Agency 3	DxD
18	AG3D3	self	Agency 3	DxD
19	AG3D4	self	Agency 3	DxD
20	AG3D5	self	Agency 3	DxD
21	AG3D6	intercross	Agency 3	DxD
22	AG3D7	self	Agency 3	DxD
23	AG3D8	intercross	Agency 3	DxD
24	AG3D9	self	Agency 3	DxD
25	AG3D10	self	Agency 3	DxD
26	AG4D1	self	Agency 4	DxD
27	AG4D2	intercross	Agency 4	DxD
28	AG5D1	intercross	Agency 5	DxD
29	AG5D2	intercross	Agency 5	DxD
30	AG6D1	intercross	Agency 6	DxD
31	AG6D2	self	Agency 6	DxD
32	AG6D3	intercross	Agency 6	DxD
33	AG6D4	self	Agency 6	DxD
34	AG6D5	intercross	Agency 6	DxD
35	AG6D6	self	Agency 6	DxD
36	AG6D7	self	Agency 6	DxD
37	AG6D8	intercross	Agency 6	DxD
38	AG6D9	self	Agency 6	DxD
39	AG6D10	self	Agency 6	DxD
40	AG3DP1	intercross	Agency 3	DxP
41	AG3DP2	intercross	Agency 3	DxP
42	AG4DP1	intercross	Agency 4	DxP
43	AG4DP2	intercross	Agency 4	DxP
44	AG5DP1	intercross	Agency 5	DxP
45	AG6DP1	intercross	Agency 6	DxP
46	AG6DP2	intercross	Agency 6	DxP
47	AG6DP3	intercross	Agency 6	DxP
48	AG6DP4	intercross	Agency 6	DxP
49	AG7DP1	intercross	Agency 6	DxP
50	AG6T1	intercross	Agency 6	TxP
51	AG6T2	intercross	Agency 6	TxP
52	AG6T3	intercross	Agency 6	TxP
53	AG6T4	intercross	Agency 6	TxP

TABLE 1. LIST OF DxD, DxP, TxT/P POPULATIONS INCLUDED IN THE STUDY (continued)

No.	Population name	Crossing scheme	Agency	Cross type
54	AG6T5	intercross	Agency 6	TxP
55	AG6T6	intercross	Agency 6	TxP
56	AG1T1	intercross	Agency 1	TxT
57	AG1T2	intercross	Agency 1	TxT
58	AG3T1	intercross	Agency 3	TxT
59	AG3T2	self	Agency 3	TxT
60	AG3T3	intercross	Agency 3	TxT
61	AG3T4	self	Agency 3	TxT
62	AG3T5	intercross	Agency 3	TxT
63	AG3T6	self	Agency 3	TxT
64	AG3T7	intercross	Agency 3	TxT
65	AG3T8	self	Agency 3	TxT
66	AG4T1	self	Agency 4	TxT
67	AG4T2	self	Agency 4	TxT

AFLP and Data Analysis

The AFLP procedure was performed with AFLP Analysis System 1 from GIBCO BRL, USA following the manufacturer's recommended protocol. Some 300 ng of each DNA sample was digested with *EcoRI* and *MseI* mixture at 37°C for 3 hr. A 24 µL of ligation mix containing *EcoRI* and *MseI* was added to each digest adaptors and used as template for pre-amplification. A 5 µL of pre-amplified DNA template, together with the [γ^{33}] ATP-labelled *EcoRI* and unlabelled *MseI* primers were subjected to selective PCR. The 8 primer combinations of *EcoRI* and *MseI* are listed in Table 2. The PCR products were electrophoresed on 6% polyacrylamide gels at 1600 V for 3 hr. Gels were exposed against X-ray films for 4-5 days in -80°C freezer.

Autoradiograms were scored manually, band presence being indicated by '1' and absence by '2'. Genetic diversity analysis was performed using PowerMarker software (Liu and Muse, 2005). Among the parameters estimated were major allele frequency, allele number, gene diversity and Polymorphic Information Content (PIC). These parameters were computed for each marker as well as population. Gene diversity refers to the probability of two randomly chosen alleles from the population are different whereas PIC measures the polymorphism degree of the markers and populations (Botstein *et al.*, 1980; Serrote *et al.*, 2020). Genetic distance among individuals was computed using Darwin software (Perrier *et al.*, 2003). Dendrograms were constructed for each DxT and TxT/P palms, following the unweighted pair group method with arithmetic mean (UPGMA) method to visualise the genetic relationship between

the palms. Principal Coordinate Analysis (PCA) was carried out to illustrate the coordinates of palms for each DxT and TxT/P crosses.

RESULTS

Genetic Diversity Revealed by AFLPs

The number of scorable bands revealed by the eight primer combinations varied between 14 and 48 (Table 2), contributing to a total of 225 bands scored for data analysis. On average, the AFLP markers recorded gene diversity and PIC values of 0.2274 and 0.2008, respectively (Table 3). The PIC of the markers ranged between 0.0628 to 0.4226 (data not shown). The genetic variability measures for each population are presented in Table 4. Among the DxT populations, the values for allele number ranged from 1.0658 to 1.6404, while that for gene diversity and PIC ranged from 0.0210 to 0.1502 and 0.0172 to 0.1292 respectively. Population AG2D1 of Agency 2 recorded the lowest score for these parameters whereas the highest was observed for population AG6D10 from Agency 6. Among the DxP populations, progeny AG6DP1 had the lowest number of alleles (1.1491), the lowest score for gene diversity (0.0467) and PIC (0.0380), while progeny AG4DP2 exhibited the highest scores for these parameters (1.5833, 0.1989 and 0.1613) respectively. As for the TxT/P crosses, gene diversity (0.0575) and PIC (0.0472) were lowest in AG1T1 but highest in AG6T6 (0.2689 and 0.2144). Progeny AG6T1 exhibited the lowest number of alleles (1.1491), while AG6T6 recorded the highest (1.1886).

TABLE 2. PRIMER COMBINATIONS USED AND THE NUMBER OF BANDS OBTAINED IN THE AFLP ANALYSIS

Primer combinations	Number of bands
E-AAC/M-CAG	32
E-ACA/M-CTT	33
E-ACC/M-CTC	15
E-ACG/M-CAA	17
E-ACT/M-CTG	29
E-ACG/M-CAT	40
E-AGG/M-CAC	14
E-AAG/M-CTA	48

TABLE 3. GENETIC DIVERSITY PARAMETERS NAMELY, MAJOR ALLELE FREQUENCY, GENE DIVERSITY AND POLYMORPHIC INFORMATION CONTENT (PIC) ESTIMATED FOR EACH PRIMER COMBINATION APPLIED IN THE STUDY

Primer combination	Major allele frequency	Gene diversity	PIC
AAC/CAG	0.8436	0.2372	0.2101
ACA/CTT	0.8229	0.2773	0.2452
ACA/CTT	0.8944	0.1758	0.1590
ACC/CTC	0.8899	0.1912	0.1752
ACG/CAA	0.7976	0.3069	0.2685
ACT/CTG	0.8690	0.2012	0.1733
AGC/CAT	0.8776	0.2026	0.1825
AAG/CAC	0.8741	0.1968	0.1737
Mean	0.8545	0.2274	0.2008

TABLE 4. GENETIC DIVERSITY PARAMETERS NAMELY, ALLELE NUMBER, GENE DIVERSITY AND POLYMORPHIC INFORMATION CONTENT (PIC) ESTIMATED FOR POPULATIONS ANALYSED IN THE STUDY

No.	Population name	Allele no.	Gene diversity	PIC
1	AG1D1	1.1579	0.0536	0.0435
2	AG1D2	1.2939	0.0903	0.0745
3	AG1D3	1.4035	0.1131	0.0944
4	AG1D4	1.3947	0.1274	0.1044
5	AG2D1	1.0658	0.0210	0.0172
6	AG2D2	1.1623	0.0419	0.0354
7	AG2D3	1.1316	0.0423	0.0344
8	AG2D4	1.2061	0.0494	0.0425
9	AG2D5	1.1842	0.0498	0.0420
10	AG2D6	1.1667	0.0512	0.0421
11	AG2D7	1.1667	0.0532	0.0436
12	AG2D8	1.2544	0.0545	0.0480
13	AG2D9	1.1886	0.0641	0.0519
14	AG2D10	1.2675	0.0740	0.0617
15	AG2D11	1.3947	0.1255	0.1037
16	AG3D1	1.1272	0.0363	0.0303
17	AG3D2	1.1316	0.0428	0.0349

TABLE 4. GENETIC DIVERSITY PARAMETERS NAMELY, ALLELE NUMBER, GENE DIVERSITY AND POLYMORPHIC INFORMATION CONTENT (PIC) ESTIMATED FOR POPULATIONS ANALYSED IN THE STUDY (continued)

No.	Population name	Allele no.	Gene diversity	PIC
18	AG3D3	1.1623	0.0494	0.0406
19	AG3D4	1.1491	0.0549	0.0438
20	AG3D5	1.1579	0.0566	0.0452
21	AG3D6	1.1886	0.0578	0.0476
22	AG3D7	1.1842	0.0587	0.0481
23	AG3D8	1.2500	0.0703	0.0590
24	AG3D9	1.3904	0.1051	0.0882
25	AG3D10	1.4167	0.1052	0.0895
26	AG4D1	1.2368	0.0738	0.0608
27	AG4D2	1.3070	0.0933	0.0772
28	AG5D1	1.2807	0.0760	0.0640
29	AG5D2	1.5395	0.1324	0.1139
30	AG6D1	1.1404	0.0436	0.0357
31	AG6D2	1.1491	0.0509	0.0411
32	AG6D3	1.1667	0.0578	0.0467
33	AG6D4	1.1842	0.0579	0.0476
34	AG6D5	1.2193	0.0786	0.0634
35	AG6D6	1.3509	0.0852	0.0729
36	AG6D7	1.2851	0.0920	0.0751
37	AG6D8	1.2807	0.1061	0.0841
38	AG6D9	1.5395	0.1469	0.1234
39	AG6D10	1.6404	0.1502	0.1292
40	AG3DP1	1.2105	0.0631	0.0519
41	AG3DP2	1.2456	0.0828	0.0668
42	AG4DP1	1.3026	0.0854	0.0713
43	AG4DP2	1.5833	0.1989	0.1613
44	AG5DP1	1.4825	0.1239	0.1053
45	AG6DP1	1.1491	0.0467	0.0380
46	AG6DP2	1.2807	0.0676	0.0581
47	AG6DP3	1.3465	0.0924	0.0776
48	AG6DP4	1.3728	0.1256	0.1032
49	AG7DP1	1.5921	0.1755	0.1456
50	AG6T1	1.1491	0.0598	0.0476
51	AG6T2	1.2719	0.1047	0.0844
52	AG6T3	1.5833	0.1617	0.1366
53	AG6T4	1.5746	0.2111	0.1688
54	AG6T5	1.4956	0.2203	0.1713
55	AG6T6	1.7149	0.2689	0.2144
56	AG1T1	1.1886	0.0575	0.0472
57	AG1T2	1.3596	0.1175	0.0961

TABLE 4. GENETIC DIVERSITY PARAMETERS NAMELY, ALLELE NUMBER, GENE DIVERSITY AND POLYMORPHIC INFORMATION CONTENT (PIC) ESTIMATED FOR POPULATIONS ANALYSED IN THE STUDY (continued)

No.	Population name	Allele no.	Gene diversity	PIC
58	AG3T1	1.2807	0.0749	0.0629
59	AG3T2	1.3202	0.0982	0.0808
60	AG3T3	1.4298	0.1045	0.0891
61	AG3T4	1.3991	0.1204	0.1002
62	AG3T5	1.3904	0.1235	0.1021
63	AG3T6	1.6623	0.1463	0.1275
64	AG3T7	1.6798	0.1796	0.1515
65	AG3T8	1.5965	0.2042	0.1648
66	AG4T1	1.3509	0.1017	0.0846
67	AG4T2	1.5570	0.1551	0.1303

The gene diversity and PIC values for DxD and TxT crosses were averaged according to their crossing schemes (intercrossing and selfing) (Figure 1). Populations derived from intercrossings showed on average higher PIC than those produced from selfings. In general, *dura* populations had lower PIC scores than those observed in TxT and TxP populations.

Population Structure Analysis

The dendrograms based on genetic distances estimated among all individual palms are presented in Figure 2 (DxD populations) and Figure 3 (TxT/P populations). Apart from the main cluster (denoted as '1'), the majority of

the DxD palms formed small sub-clusters, not according to genetic background or agency. In the main cluster, two sub-populations were observed; the first was confined to DxD palms from Agency 2 only while the second sub-cluster consisted of palms from six agencies. The palms of the TxT/P populations also formed several sub-clusters but each sub-cluster consisted of palms from the same agency (Figure 3a). The TxT/P palms attained from Agency 3 formed sub-clusters with mixed origins. The Principal Component Analysis (PCA) of the DxD (Figure 4a) and TxT (Figure 4b) revealed that the majority of palms from different agencies, derived from various genetic backgrounds were positioned at the centre of the PCA, signifying a close genetic relationship.

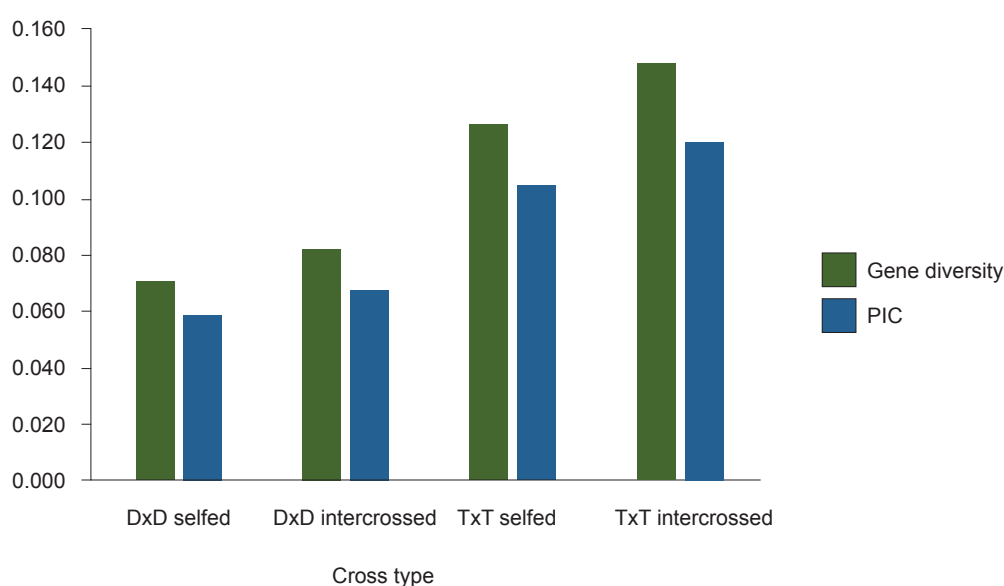


Figure 1. Barplots showing the differences in gene diversity and PIC values between selfed and intercrossed populations for both DxD and TxT/P crosses. In general, selfed population exhibited lower diversity values compared to intercrossed.

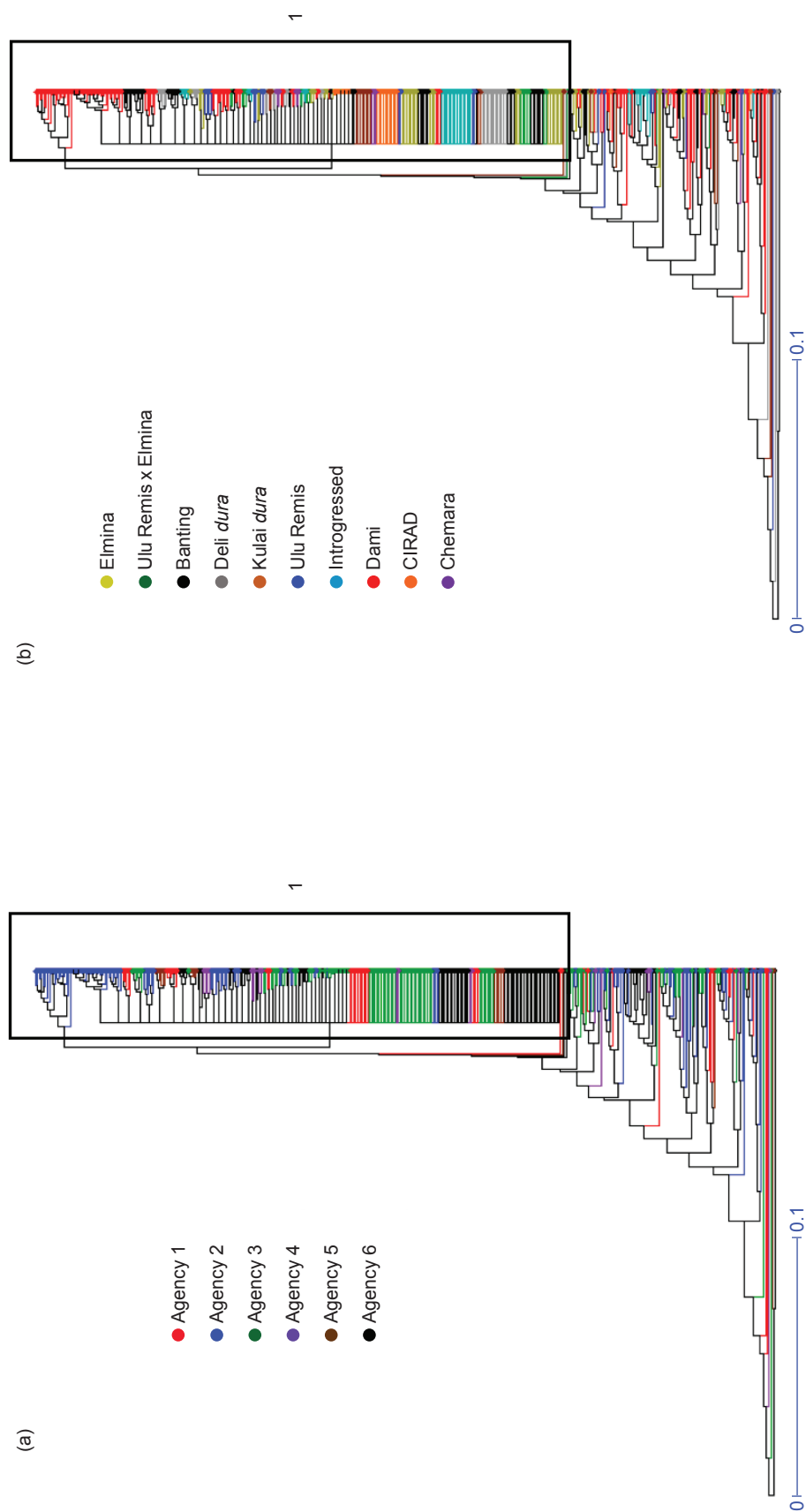


Figure 2. Dendrograms presenting the genetic relationship between DxD individual palms analysed in the study. Each branch represents individual palm, coloured respectively according to (a) six oil palm agencies, (b) genetic backgrounds. The main cluster (marked as 1) is composed of palms from various agencies. The sub-populations observed at the bottom contained palms from mixed agencies and genetic backgrounds.

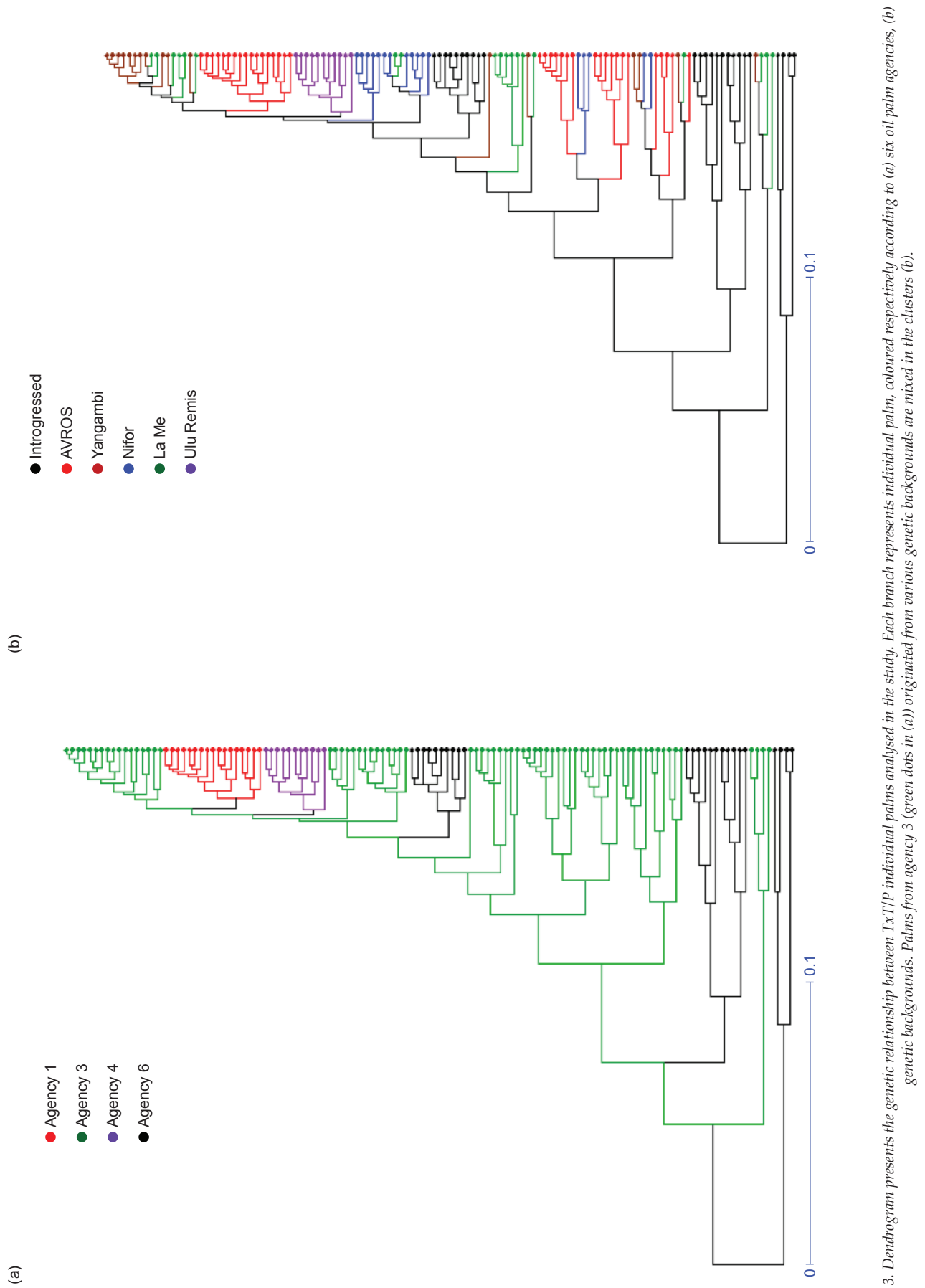


Figure 3. Dendrogram presents the genetic relationship between TxT/P individual palms analysed in the study. Each branch represents individual palm, coloured respectively according to (a) six oil palm agencies, (b) genetic backgrounds. Palms from agency 3 (green dots in (a)) originated from various genetic backgrounds are mixed in the clusters (b).

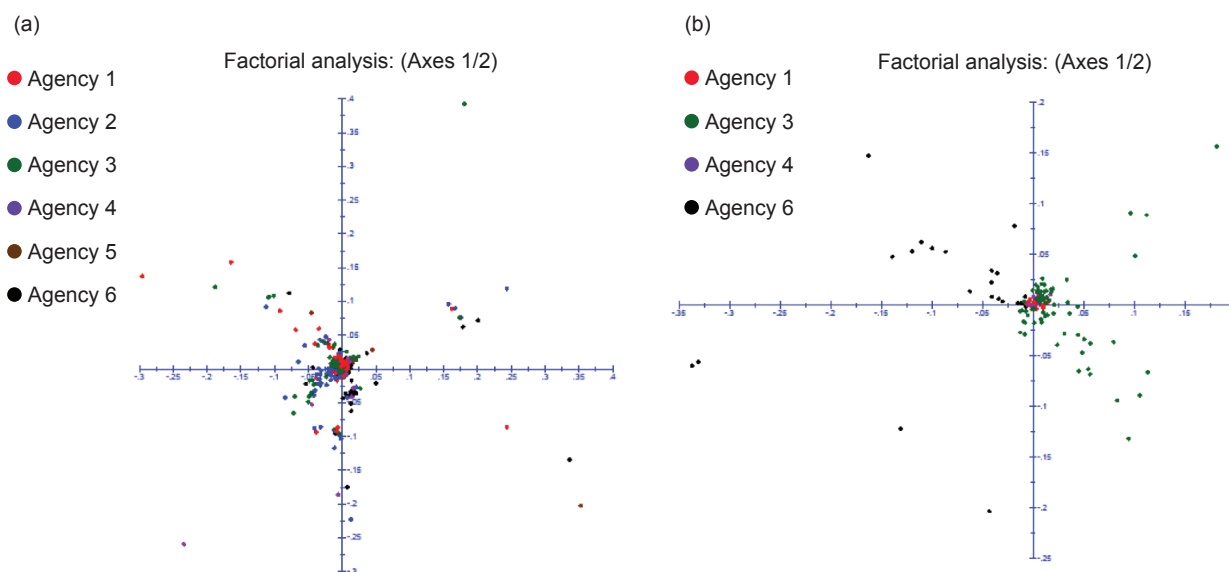


Figure 4. Principal Component Analysis showing coordinates of (a) DxT and (b) TxT individual palms. Dots are coloured corresponding to the six oil palm agencies. Most palms are located close to the centre of PCA. No clear groupings are observed.

DISCUSSION

The AFLP technique, which has proven reliable in plant genetic studies over the last 20 years, was utilised to obtain an overview of the diversity of the advanced oil palm breeding populations in Malaysia. In this study, although on average, the AFLP markers exhibited moderate levels of informativeness ($PIC = 0.2008$), there are markers that recorded PIC above 0.3000, signifying high discriminatory power for determining the diversity of the breeding populations. However, as AFLP is a dominant marker, it is not able to detect recessive alleles or select for palms that are homozygous or heterozygous at a particular locus for breeding purposes. Despite these limitations, the AFLP markers provided useful genetic information for oil palm genetic and breeding studies. AFLP markers can be linked to specific loci under selection (Jump and Penuelas, 2007) and Kirschner *et al.* (2021) were of the view that since the AFLP technique can analyse a large number of loci for polymorphism with a single primer pair, it is comparable to Restriction-Site Associated DNA (RAD) sequencing in its effectiveness at genetic analysis.

Oil palm breeding and improvement programmes in Malaysia utilised genetic materials from restricted sources. The impact of selection and breeding on breeding populations with narrow genetic bases can be more prominent as recombination involving recessive alleles or undesirable genes can affect population fitness (Rosenquist, 1986), indicating the importance of continuously evaluating the genetic diversity of the oil palm advanced breeding populations. In this

study, oil palm breeding populations originating from various genetic backgrounds provided by six oil palm research agencies were analysed. The phylogenetic tree of the DxT individual palms displayed one main cluster and several sub-clusters with no clear preference to genetic background or agency. The PCA showed that majority of the palms were positioned at the center, demonstrating genetic similarity, except a few that were outside the cluster suggesting that the DxT palms analysed had close genetic relationship. This finding was similar to those reported in previous analyses on oil palm breeding populations (Budiman *et al.*, 2019; Chun *et al.*, 2018; Purba *et al.*, 2000).

It would appear that the *dura* breeding populations at the different agencies had very similar genetic background. Although the populations analysed in this study were from different selection programmes (e.g., Banting, Kulai *dura*, Dami, Elmina Chemara, Ulu Remis), they nevertheless originated from the four Bogor palms established at the Bogor Botanical Garden, Indonesia in 1848. The narrow gene pool among the *dura* populations had resulted in the accumulation of a mostly common set of alleles despite different selection pressure imposed by the agencies in their breeding programmes. This also contributed to the lower average gene diversity and PIC in the DxT compared with those of the TxT/P populations. Low diversity among breeding lines has also been observed in breeding programmes of other plant species such as cauliflower (Lee *et al.*, 2020), cucumber (Zhu *et al.*, 2018), cacao (Aikpokpodion *et al.*, 2009), conifer (*Platycladus orientalis*) (Jin *et al.*, 2016), sunflower (Filippi *et al.*, 2020) and cotton (Billings *et al.*, 2021).

The dendrogram for the TxT/P palms generally displayed grouping according to the agency. The *tenera* populations in Malaysia originated from several African breeding programmes. Thus, when subjected to breeding and selection, distinctive sets of alleles likely accumulated in materials sourced from the different agencies. It was observed that the populations from Agency 3 are mixed across several clusters. Agency 3 had likely sourced materials of different genetic backgrounds for incorporation into their breeding programme as part of the initiative to develop planting materials with slower vertical growth, bigger fruit bunches and larger fruits. Similar to the Dx/D palms, the TxT/P palms were also positioned at the centre of the PCA plot signifying the need for introducing new genetic resources. Although the global oil palm industry had benefited from hybrid vigour, sustaining variability among the parental lines is essential for further genetic improvement.

The conventional breeding programme involves improving the maternal *dura* and paternal *pisifera* lines separately where the parental lines are subsequently hybridised via controlled pollination to realise the hybrid vigour in the commercial DxP seedlings. The two breeding schemes employed are reciprocal recurrent selection (RSS) and modified reciprocal recurrent selection (MRRS), depending on the overall objective of the improvement programme. The RRS scheme often involves selfing of palms prior to seed production, as a means to improve the homozygosity of parental lines and reduce variation of subsequent commercial progenies. The MRRS scheme although accommodates selfing, intercrossing of selected lines is widely practised in parental improvement programmes. As expected, the study showed that populations derived from intercrossing recorded relatively higher diversity than selfing. It seems that selfing is inducing homozygosity as intended, which is in maize inbred lines and can contribute towards hybrid vigour when outcrossed to selected *pisifera*. Crossing between palms from Dx/D and TxT/P populations that recorded high homozygosity, indicated by low gene diversity and PIC values estimated in this study, can likely result in higher uniformity in the resulting hybrids as desired by commercial estates. However, intercrossing as largely practised in the MRRS scheme in Malaysia, can help maintain diversity within the parental improvement programme, especially if the breeders select and hybridise palms from specific populations that record high genetic distance values. These resulting parental populations will thus possess a higher number of different alleles, thus hybridisation between them would result in hybrids with relatively higher genetic diversity. Sritharan *et al.* (2017) had shown that hybrids generated from more diverse parental backgrounds can result in higher oil yield than those

from a limited and highly inbred genetic background. In fact, the breeders have also started incorporating genetic source from the Nigerian germplasm as a pollen source to enhance diversity and generate new planting materials known as PS1.1 that showed high yield with slow height increment (Arolu, 2017; Kushairi *et al.*, 2001; Kushairi and Amiruddin, 2020). The outcome from such an approach can reduce the risk of inbreeding depression as well as safeguard genetic variability for selection gain in oil palm in the future. In summary, marker-assisted efforts can help select palms for crossing to realise the desired hybrid vigour while also assisting in increasing variability among breeding populations, in the effort to develop more resilient varieties against diseases and climate change. The overview of the advanced breeding populations in Malaysia obtained via AFLP in this study can be further complemented by other co-dominant markers such as SSR and SNPs in the future.

CONCLUSION

AFLP marker analysis revealed high genetic similarity among selected oil palm (*Elaeis guineensis* Jacq.) breeding populations utilised in Malaysia, due to the narrow gene pool. The introduction of new genetic resources into the *dura* and *tenera/pisifera* populations is highly recommended. Such efforts can help increase genetic variability among the breeding populations to ensure selection gain and maintain survival against diseases and extreme climate change in the future.

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INITIAL CHARACTERISATION OF *Metarhizium anisopliae* CPMa1502 FOR THE DEVELOPMENT OF A BIOPESTICIDE AGAINST THE OIL PALM FRUIT SCRAPER *Demotispa neivai* (Coleoptera: Chrysomelidae)

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ABSTRACT

Entomopathogenic fungi are key components of biological pest control programs. Among these, *Metarhizium anisopliae* is one of the best-studied and has been demonstrated to be particularly effective against coleopterans. The native *M. anisopliae* CPMa1502, previously selected for its insecticidal activity against *Demotispa neivai* (oil palm fruit scraper), was studied to establish its growth parameters under several culture conditions and its virulence on different stages of *D. neivai*. Considerable conidia production of CPMa1502 on MAYP (Maltose Agar Yeast with Potato Extract) and SMAYR (Sabouraud Maltose Agar with yeast extract and rice extract) agar was observed (1×10^9 conidia cm^{-2} at 14 days). The isolate was highly tolerant to a wide range of pH (5-9; germination >90%; radial growth rate: 0.95-1.02 mm day^{-1}) but moderately tolerant to high temperatures (>35°C). Additionally, the adhesion capacity to *D. neivai* cuticle and conidial hydrophobicity was strong (114 conidia mm^{-2} ; 72%). Finally, the insecticidal activity of CPMa1502 was greater on larvae than on adults, with a mean lethal concentration (LC_{50}) of 1.8×10^4 conidia mL^{-1} and a mean lethal time (LT_{50}) of 4.3 days, than on adults. These findings show that CPMa1502 is a promising candidate for further development as a biopesticide against *D. neivai*.

Keywords: biocontrol activity, conidia production, *Elaeis guineensis*, fruit scraper, growth parameters.

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INTRODUCTION

Entomopathogenic fungi have been well studied and developed into biopesticides, due to their

high virulence towards a wide range of insect pests, their ability to form resistant structures and persist in the environment, and their innocuity to the environment and human health (Zimmermann, 2007). The most studied entomopathogenic fungi belong to the genus *Metarhizium* (Meyling and Eilenberg, 2007; Zimmermann, 2007). *Metarhizium anisopliae* has been isolated from insect cadavers, soil, and roots. It synthesises various compounds (lipases, adhesives, proteases, and chitinases) to degrade insect cuticle (Schrank and Vainstein, 2010), allowing it to grow hyphae that colonise the hemolymph, leading to insect death and sporulation (Gul *et al.*, 2014; Zimmermann, 2007). *Metarhizium anisopliae* has been shown to be particularly active against Coleoptera, making it

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a prime candidate to control various pest beetles (Batta, 2004; Erler and Ates, 2015; Hajek *et al.*, 2007).

Oil palm (*Elaeis guineensis* or the hybrid *E. oleifera* x *E. guineensis*) is one of the most important oleaginous agricultural crops in the world. In Colombia, there are currently more than 559 000 cultivated hectares of this crop, making it the fourth-largest producer in the world. Like other agricultural crops, oil palm is seriously affected by various insect pests and entomopathogenic fungi have been used effectively in their control. For instance, entomopathogenic fungi have been shown to have high efficacy for the control of palm pests such as *Metisa plana* (Walker) (mortality of more than 95.0% with *Beauveria bassiana*), *Oryctes rhinoceros* (Scarabaeidae) (mortality of 100% with *M. anisopliae*), *Pteroma pendula* (mortality of 93.8% with *Paecilomyces carneus*), *Stenoma impressella* (mortality from 61.0% to 96.0% with *Cordyceps cateniannulata*), *Coptotermes curvignathus* (mortality from 71.0% to 84.0% with *M. anisopliae*), and *Demotispia neivai* (mortality from 68.0% to 95.0% with *M. anisopliae*) (Bakeri *et al.*, 2009; Kin *et al.*, 2017; Montes-Bazurto *et al.*, 2020a; 2020b; Ramle *et al.*, 2006; Ramle and Basri, 2004).

The oil palm fruit scraper, *D. neivai* Bondar (Coleoptera: Chrysomelidae), is an economically impactful emerging pest in oil palm plantations in Colombia. Both larvae and adults feed on the epidermis of fruits causing superficial damage and changing fruit colour. Insect feeding affects fruit development resulting in losses of approximately 7% in oil extraction. Furthermore, the superficial damage on the bunches makes it difficult to determine fruit maturity in the field, causing an additional 8% fruit loss (Aldana *et al.*, 2004; Valencia *et al.*, 2007). A recent study demonstrated that a native isolate of *M. anisopliae* (CPMa1502) caused up to 95% mortality in *D. neivai* larvae and adults under laboratory conditions (Montes-Bazurto *et al.*, 2020b) and 62% larval mortality under field conditions (Montes-Bazurto *et al.*, 2019). These findings demonstrated a marked biocontrol potential of CPMa1502 for further development as the first biological pesticide against *D. neivai* in Colombia. Fungal biopesticides require that their active ingredients fulfil minimal requirements for production and commercialisation from early research stages, such as conidia yield, growth parameters, and virulence. Therefore, the aim of this study was to assess desirable attributes such as growth conditions (production media, temperature, and pH), virulence factors (adherence and hydrophobicity), and insecticidal activity (lethal concentrations and mean lethal time) of *M. anisopliae* CPMa1502.

MATERIALS AND METHODS

Fungal Isolate and Reactivation

Metarhizium anisopliae isolate CPMa1502 (MH673410, MH698453 - GenBank), was obtained from the Entomopathogen Collection of Cenipalma (Montes-Bazurto *et al.*, 2020b). The fungus was originally isolated from *D. neivai* larvae, collected from oil palm plantations in San Vicente de Chucurí, Santander, Colombia (Montes-Bazurto *et al.*, 2019). It was reactivated on *D. neivai* adults and then grown on Maltose Agar Yeast with 0.5% v/v Potato Extract (MAYP) (Edelstein *et al.*, 2004), supplemented with 0.1% w/v chloramphenicol (Colmed® International, Colombia), and incubated at 25°C ± 2°C for 14 days in total darkness. Conidia were harvested from Petri plates by carefully scraping the surface with a sterile scalpel.

Growth Parameters

Conidia production. CPMa1502 conidia obtained in MAYP agar were suspended in a 0.1% Tween® 80 solution (Merck, Germany), and the concentration was adjusted to 1x10⁶ conidia mL⁻¹ using a hemocytometer. Subsequently, eight agar media reported for *M. anisopliae* conidia production were inoculated with 0.1 mL of the conidial suspension: 1. Potato Dextrose Agar (PDA) (Gandarilla-Pacheco *et al.*, 2012); 2. PDA with 0.5% peptone (PDAP) (Gandarilla-Pacheco *et al.*, 2012); 3. PDA with 0.5% yeast extract (PDAE) (Gandarilla-Pacheco *et al.*, 2012); 4. Yeast Malt Agar (YM); 5. MAYP (Edelstein *et al.*, 2004); 6. Sabouraud Dextrose Agar (SDA); 7. Sabouraud Maltose Agar with 1% yeast extract (SMAY) (Edelstein *et al.*, 2004); and 8. SMAY supplemented with 13% rice extract (SMAYR) (Edelstein *et al.*, 2004). The culture media were then incubated at 25°C ± 0.5°C (Memmert, Germany) for 10, 12 and 14 days. At each time, the growing biomass was harvested and suspended in 10 mL 0.1% Tween® 80 solution. The conidia concentration of each suspension was assessed to determine sporulation or conidia yield per unit area (conidia cm⁻²). The conidia production test was carried out in triplicate for each treatment (growth media* incubation time), and the whole experiment was replicated three times.

Growth temperature. CPMa1502 conidia were suspended in a 0.10000% Tween® 80 solution, and the concentration was adjusted to 1x10⁸ conidia mL⁻¹ using a hemocytometer. Germination was assessed by transferring 100 µL of dilution 10⁻² of the conidial suspension to Petri plates with YM agar with 0.00004% benomyl (Benlate, DuPont, Spain). Plates were incubated at six temperatures for 16 and 24 hr (Faria *et al.*, 2015): 20°C ± 0.5°C (Binder,

Germany); 25°C ± 0.5°C (Mettler, Germany); 30°C ± 1°C (Sheldon Manufacturing, Inc., USA); 35°C ± 1°C (Binder, Germany); 40°C ± 2°C (Mettler, Germany); and 45°C ± 2°C (Mettler, Germany). An agar square of 1 cm² was removed and then a drop of lactophenol blue solution was added (Merck, Germany). Germinated and non-germinated conidia were determined using an optical microscope (Olympus®, Japan), and 100 conidia were scored per replicate. A germinated conidium must have a germ tube length of at least twice the conidia diameter (Ekesi *et al.*, 1999).

The radial growth rate was determined by measuring colony radius daily in a Petri plate with MAYP agar with 0.1% w/v chloramphenicol, inoculated with 10 µL of a conidial suspension in the middle of the plate at the six temperatures mentioned above for 14 days and reported as mm day⁻¹. The growth temperature test was carried out in triplicate with three replicates per repetition.

Effect of pH. A conidial suspension at 1x10⁸ conidia mL⁻¹ was prepared to inoculate 15 mL glass flasks with phosphate buffer adjusted to pH 5, 6, 7, 8 and 9 [1:9/conidia: buffer (Merck, Germany)], as liquid culture media. Sterile distilled water was used as a control (pH=5.88 ± 0.45). The flasks were incubated on a heated orbital shaker at 150 rpm, 25°C ± 1°C for 24 hr (Heidolph, Germany). Subsequently, germination and radial growth were measured, as described above, at a single incubation temperature of 25°C ± 0.5°C. The test was repeated three times and replicated three times.

Conidial Adherence and Relative Hydrophobicity

Conidial adherence was determined based on the methodology developed by Boucias *et al.* (1988). Cuticle ghosts were prepared from *D. neivai* adults by dipping them in 15% KOH (Merck, Germany), and boiling until the fragments became translucent. Cuticle ghosts were then mixed in a conidial suspension of *M. anisopliae* CPMa1502, adjusted to 1x10⁷ conidia mL⁻¹ and maintained at 200 rpm for 1 hr at room temperature (ThermoFisher Scientific, USA). Subsequently, the mixture was filtered with a non-woven fabric to recover the cuticles which were then cleaned with sterile distilled water. The conidia attached to the cuticle (1 mm²) were revealed with lactophenol blue (Merck, Germany) and counted using a hemocytometer. The results were reported as conidia per unit area of the *D. neivai* cuticle.

Hydrophobicity assays were carried out according to the phase exclusion methodology described by Boucias *et al.* (1988) and Shah *et al.* (2007). Briefly, 10 mg of dry conidia were suspended in a 0.1M KNO₃ solution (aqueous phase; Merck, Germany) and absorbance was read at 600 nm

(Mecasys, Korea). A volume of 3 mL of the aqueous phase was then mixed with 1 mL of toluene (organic phase; Merck, Germany), gently vortexed for 20 s, and left to rest for 30 min. The aqueous phase was extracted and absorbance was assessed at 600 nm. Relative hydrophobicity percentage (*H*) was calculated using the following Equation (1):

$$H (\%) = \frac{A_0 - A_f}{A_f} \times 100 \quad (1)$$

where *H* is the relative hydrophobicity, *A*₀ is the initial absorbance of the aqueous phase, and *A*_f is the absorbance of the aqueous phase after mixing with toluene.

Conidial adherence and relative hydrophobicity were assessed three times, with nine measurements carried out for adherence and three for hydrophobicity.

Insecticidal Activity

Insects. *Demotista neivai* adults and larvae used to establish the rearing or for bioassays were obtained from oil palm fruit bunches, collected on a naturally infested oil palm plot, located near Cenipalma's Palmar de la Vizcaína research station, Santander, Colombia. Collected individuals were subjected to a 10 day quarantine to avoid contamination by naturally occurring pathogens or parasitoids. Adults and larvae were placed in 8 × 5 cm PVC tubes, closed at both ends with muslin and allowed to feed and oviposit on ripe fruits. The eggs found on fruits were individualised and monitored until hatching. Ripe palm fruits were replaced as necessary. Insects were kept at 27°C ± 1°C, 90% ± 5% relative humidity (RH), and a photoperiod of 12:12 hr (light: dark), until they were needed for bioassays.

Concentration-mortality and time-mortality tests.

The lethal concentrations for CPMa1502 conidia, grown on MAYP agar, were determined on adults and larvae of *D. neivai*, using laboratory bioassays (Montes-Bazurto *et al.*, 2020b). For the adult bioassay, adults of *D. neivai* were immersed for 1 min in an aqueous suspension of *M. anisopliae* CPMa1502 conidia, adjusted to five different concentrations (1x10⁴, 1x10⁵, 1x10⁶, 1x10⁷, and 1x10⁸ conidia mL⁻¹), in 0.1% Tween® 80 solution. For control treatments, a solution of 0.1% v/v Tween® 80 was used, whereas, for absolute controls, insects were untreated. Larvae were inoculated by placing a 15 µL drop of conidial suspension on the thorax, adjusted to the same concentrations as those used in the adult bioassay. After inoculation, insects were maintained individually in 8 cm diameter × 5 cm PVC tubes covered with muslin on both ends at 27°C ± 1°C and 90% ± 5% RH, under a natural photoperiod on oil

palm fruits. Insect mortality was recorded every day for 15 days. Mortality data for treatments was corrected with mortality data from the control, to determine the mean lethal concentrations LC_{50} and lethal concentration ninety LC_{90} were determined using Probit analysis (Finney, 1952). The lethal time (LT_{50}) was calculated using the mortality data of the pathogenicity bioassays for the treatment corresponding to a fungal concentration of 1×10^8 conidia mL^{-1} for both adults and larvae.

A completely randomised design was used with five replicates per treatment (conidia concentration) for adult bioassay ($n=5$) and three per treatment for larvae bioassay ($n=3$). Each replicate consisted of 10 individual insects (experimental units) for a total of 50 adults, and 30 larvae used per treatment. Different methods were used for larval and adult bioassays since larvae drowned when the immersion method was used and drops rolled off adult elytra.

Statistical Analysis

For statistical purposes, germination was arcsine or square-root transformed. Statistical significance of treatments was determined using one-way analysis of variance (ANOVA), and mean separations were compared using Tukey HSD's test with a confidence level of 95%. Analysis was carried out using Statistix® version 8.1 (Analytical Software

2003). Probit analysis (Finney, 1952) was used to determine the lethal concentrations using POLO-PLUS 1.0 (LeOra Software 2006) and lethal time data were subjected to Weibull survival analysis using the Generalised Linear Interactive Modeling Program (Crawley, 1993).

RESULTS AND DISCUSSION

Growth Parameters

Conidia production. All the media assessed allowed the growth and sporulation of CPMa1502. The fermentation started with the colonisation of white mycelium and the production of dark green aerial conidia after 6-7 days of incubation. The conidia yields at 10, 12 and 14 days are presented in Figure 1a. Sparse sporulation was observed in PDAP, PDAE, YM, and SDA agar, followed by PDA and SMAY media (Figure 1b). MAYP and SMAYR reached the highest conidia yields at all three times evaluated. Significant differences were found between the eight culture media on the 10th and 12th day of growth (10th day: $F_{7,16}=4.93$, $p=0.0039$; 12th day: $F_{7,16}=4.32$, $p=0.0073$); while no statistical differences were observed on the 14th day ($F_{7,16}=2.11$; $p=0.1023$). The largest yield was reached on the 14th day using MAYP and SMAYR (MAYP: $1.1 \pm 0.4 \times 10^9$ conidia cm^{-2} ; SMAYR: $1.1 \pm 0.3 \times 10^9$ conidia cm^{-2}).

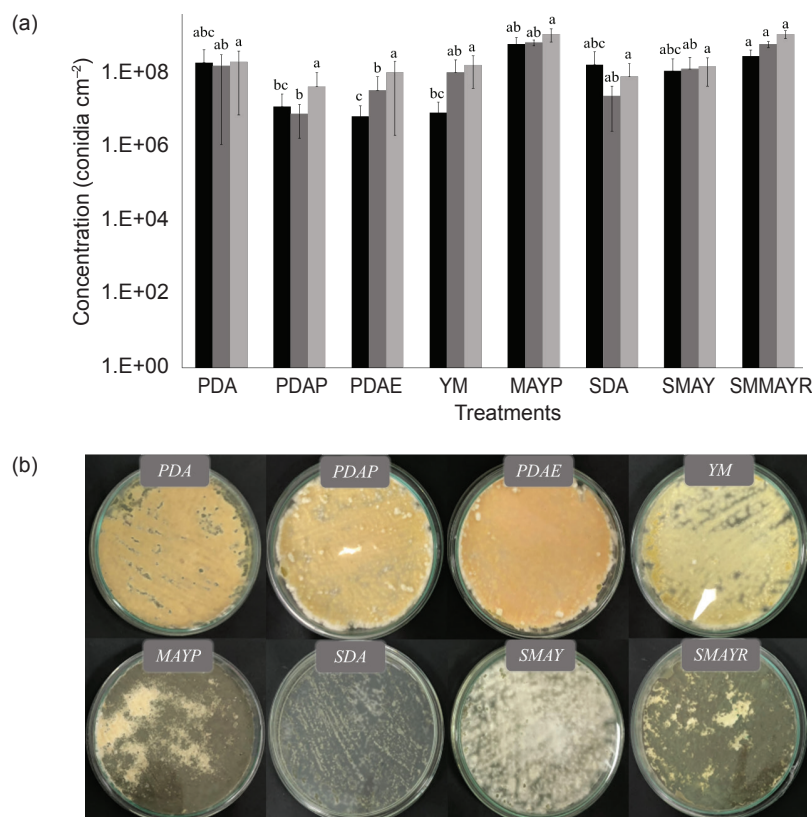


Figure 1. Conidia production of *M. anisopliae* CPMa1502 on different agar media. (a) Conidia concentration (conidia cm^{-2}) at 10(■), 12(■), and 14(■) days incubation; (b) In vitro growth at 14 days. Values with different lowercase letters show statistical differences according to Tukey HDS Test ($p<0.05$).

CPMa1502 achieved the highest yield on MAYP and SMAYR agar, and which could be attributed to the sources of carbon, nitrogen, proteins, vitamins, and amino acids favoring the conidiogenesis process. Yeast extract has been identified as a sporulation inductor, and enables increased biomass production due to its vitamin B content (Patil *et al.*, 2014). Also, the starches used in these media can also induce the enzymatic capacity for the degradation of polysaccharides, similar to insect cuticles, and activate several virulence mechanisms (Clarkson and Charnley, 1996; Permadi *et al.*, 2020). The yields achieved were 10 times higher than the values reported for *Metarhizium* sp., using YM agar (7.7×10^6 conidia cm^{-2}) (Aguirre *et al.*, 2009). MAYP has also been found to favour biomass production and increase the growth rate for other species of *Metarhizium* (Edelstein *et al.*, 2004).

Growth temperature. Germination after 16 and 24 hr was greater than 85% for temperatures between 20°C–35°C, and was no different (16 hr: $F_{5,48}=149$, $p<0.0001$; 24 hr: $F_{5,48}=101$, $p<0.0001$) (Table 1). There was a 3%–24% decrease in germination after 16 hr of incubation, and 9%–18% at 24 hr at 40°C $\pm$ 2°C of incubation, compared with the values found at 20°C–35°C (16 hr: $F_{3,32}=11.6$, $p<0.0001$; 24 hr: $F_{3,32}=6.06$, $p=0.0007$). Furthermore, at 45°C $\pm$ 2°C, the reduction at both times ranged between 85% and 98% (16 hr: $F_{3,32}=165$, $p<0.0001$; 24 hr: $F_{3,32}=115$, $p<0.0001$). Radial growth was not affected by the temperature range between 20°C to 35°C. The growth rates at 40°C $\pm$ 2°C and 45°C $\pm$ 2°C were

significantly lower than the findings at 20°C–35°C, and no growth was observed at 45°C ($F_{5,48}=352$, $p<0.0001$).

The high-temperature tolerance of *M. anisopliae* CPMa1502 conidia will enable its persistence in the environment, despite daily thermal fluctuations in tropical regions for longer periods, ensuring its effectiveness in pest control. Thermotolerant conidia will also be able to resist formulation processes, such as drying and storage at high temperatures (30°C–40°C) (Alves *et al.*, 2017; Athanassiou *et al.*, 2017; Oliveira *et al.*, 2016; Rangel *et al.*, 2005). *Metarhizium anisopliae* CPMa1502 showed optimal growth at a temperature range between 20°C and 35°C, and no appreciable decrease in germination was observed up to 40°C after 24 hr (>80%). High viability at temperatures above 25°C could be due to the geographical region where this microorganism was isolated, which has a maximum temperature of 40°C (IDEAM, 2018; 2019). Other *Metarhizium* sp. isolated in tropical regions showed a similar response, suggesting a direct relationship between the isolates' geographical area and their tolerance to temperature (Fargues *et al.*, 1997; Fernandes *et al.*, 2010; Hallsworth and Magan, 1996; 1999; Rangel *et al.*, 2005).

Effect of pH. CPMa1502 germination (16 and 24 hr) was above 95% for the range of pH evaluated and no differences were observed (16 hr: $F_{5,48}=0.94$; $p=0.4660$; 24 hr: $F_{5,48}=0.68$; $p=0.6421$) (Table 2). Similarly, pH did not affect the radial growth rate as shown in Figure 2 ($F_{3,32}=0.39$, $p=0.8451$).

TABLE 1. EFFECT OF GROWTH TEMPERATURE ON CONIDIAL GERMINATION AT 16 hr AND 24 hr, AND RADIAL GROWTH RATE (mean $\pm$ SD)

Temperature (°C)	Germination at 16 hr (%)	Germination at 24 hr (%)	Radial growth rate (mm day ⁻¹)
20 $\pm$ 0.5	89.5 $\pm$ 1.5 ^a	95.4 $\pm$ 1.1 ^a	1.14 $\pm$ 0.08 ^a
25 $\pm$ 0.5	89.5 $\pm$ 3.5 ^a	93.1 $\pm$ 0.9 ^a	1.07 $\pm$ 0.17 ^a
30 $\pm$ 1.0	89.6 $\pm$ 2.5 ^a	92.6 $\pm$ 1.8 ^a	1.13 $\pm$ 0.15 ^a
35 $\pm$ 1.0	89.3 $\pm$ 1.2 ^a	93.5 $\pm$ 0.6 ^a	1.16 $\pm$ 0.10 ^a
40 $\pm$ 2.0	78.5 $\pm$ 1.8 ^b	82.7 $\pm$ 1.4 ^b	0.12 $\pm$ 0.01 ^b
45 $\pm$ 2.0	3.9 $\pm$ 0.3 ^c	5.9 $\pm$ 1.6 ^c	0.00 $\pm$ 0.00 ^c

Note: Values with different lowercase letters show statistical differences according to Tukey HDS Test ($p<0.05$).

TABLE 2. EFFECT OF pH ON CONIDIAL GERMINATION AT 16 hr AND 24 hr, AND RADIAL GROWTH RATE (mean $\pm$ SD)

pH	Germination at 16 hr (%)	Germination at 24 hr (%)	Radial growth rate (mm day ⁻¹)
Control	98.2 $\pm$ 1.4 ^a	99.1 $\pm$ 1.3 ^a	0.97 $\pm$ 0.04 ^a
5	96.2 $\pm$ 4.3 ^a	98.5 $\pm$ 2.2 ^a	0.95 $\pm$ 0.07 ^a
6	97.6 $\pm$ 1.7 ^a	98.9 $\pm$ 1.6 ^a	0.99 $\pm$ 0.06 ^a
7	96.7 $\pm$ 2.7 ^a	98.1 $\pm$ 1.8 ^a	1.02 $\pm$ 0.11 ^a
8	95.3 $\pm$ 4.4 ^a	97.4 $\pm$ 3.5 ^a	1.00 $\pm$ 0.09 ^a
9	96.4 $\pm$ 3.8 ^a	97.1 $\pm$ 3.2 ^a	0.99 $\pm$ 0.07 ^a

Note: Values with different lowercase letter show statistical differences according to Tukey HDS Test ($p<0.05$).

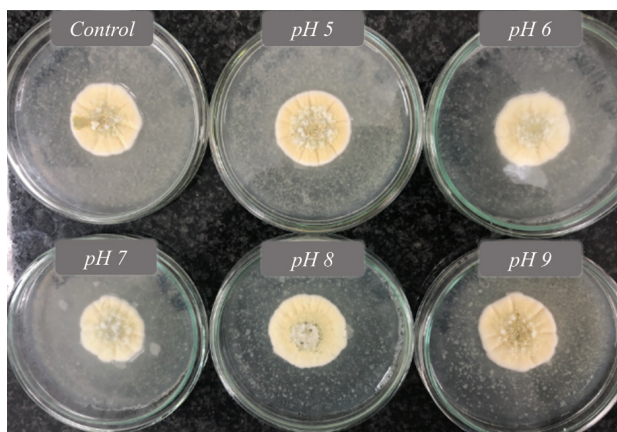


Figure 2. Effect of pH on the radial growth rate of *M. anisopliae* CPMa1502.

Tolerance to varying pH is also relevant since it facilitates the mass production of the fungi and subsequent formulation processes. Moreover, in-field applications are more reliable in pH-tolerant strains since the pH of water used in the field varies. This could reduce the efficacy of pH-sensitive isolates by altering cell structures and modifying micronutrient assimilation, resulting in changes to cell morphology and duplication (Glazebrook *et al.*, 1992; Liu *et al.*, 2007; Tamerler *et al.*, 1998). Previous studies have shown that *M. anisopliae* can grow in pH range between 2.5 to 10.5 (Hallsworth and Magan, 1996; St Leger *et al.*, 1999). Its ability to grow in acidic and basic environments is due to its ability to modulate the cytosolic production of organic acids to maintain pH homeostasis (St Leger *et al.*, 1999).

Conidial Adherence and Relative Hydrophobicity

Groups of more than 10 conidia were observed in some areas of the host cuticle without showing a specific distribution pattern, affecting the CV>20%. Despite this, the mean conidial adherence was 114 ± 36 conidia mm⁻² without significant differences among repetitions ($F_{2,24}=0.68$, $p=0.5172$). Mean relative hydrophobicity was near 72%, with a CV<10% ($F_{2,6}=2.38$, $p=0.1735$).

Conidial adherence and hydrophobicity are critical factors that determine the interaction between fungal propagules and their host insects, and fungal dispersion (Jeffs *et al.*, 1999; Sevim *et al.*, 2012). Fungal propagules have different adhesion properties as a response to adaptation to environmental conditions. Aerial conidia tend to adhere strongly to hydrophobic surfaces, while submerged conidia can adhere to both hydrophilic and hydrophobic surfaces (Holder and Keyhani, 2005). Additionally, the high adherence to the fungal conidia allows for the induction of a continuous infection (Staples and Milner, 2000). The adherence of *M. anisopliae* CPMa1502 conidia to *D. neivai* cuticle represented 11% of the initial conidia concentration

(1×10^7 conidia mL⁻¹). The conidia adhered irregularly to the insect, and preferred areas of the host cuticle with tiny spines or other irregularities, leading to high variability in adherence (Boucias *et al.*, 1988). Fernandez *et al.* (2001) assessed the number of conidia adhered to different parts of the insect body with diverse inoculation methods, and found a higher conidia density on the dorsal surface compared to other parts of insects, regardless of the inoculation method. Villamizar and Cotes (2003) reported similar findings using *M. anisopliae* conidia obtained from submerged fermentation; when the authors assessed the adherence with conidia from solid-state fermentation, the values increased between three and six fold. Therefore, each type of propagule has different adhesion and hydrophobicity properties as a response to adaptation to environmental and nutritional conditions.

High hydrophobicity is also a helpful characteristic due to the interaction between amphipathic proteins (hydrophobins) involved in conidia host and fruits interactions since both the waxy surface of oil palm fruits and the lipid layer of the insect cuticle is hydrophobic (Dubey *et al.*, 2014). Similar to adherence, conidia hydrophobicity can be influenced by the nutritional composition of the media (Holder and Keyhani, 2005; Rosenberg *et al.*, 1980). A comparable hydrophobicity of *M. anisopliae* CPMa1502 was reported by Jeffs *et al.* (1999) using *M. anisopliae* isolates, showing hydrophobicities between 65%-69% using a salt-mediated aggregation and sedimentation assay (SAS). Luke *et al.* (2014) observed a relative hydrophobicity between 62%-71% with the SAS assay, and 95%-98% with the phase exclusion method for four *Metarhizium* species. Villamizar and Cotes (2003) achieved comparable values of relative hydrophobic with conidia recovered from solid-state fermentation. Boucias *et al.* (1988) found a relative hydrophobicity of 100% in *M. anisopliae* conidia grown on a synthetic medium.

Insecticidal Activity

Concentration-mortality test. Mortality of adults and larvae of *D. neivai* was demonstrated at different fungal concentrations and oscillated between 4.0% and 78.0% in *D. neivai* adults and between 51.9% and 100% in larvae (Figure 3). There were significant differences between the different concentrations evaluated in the mortality of both adults ($F_{5,24}=13.90$, $p<0.0001$) and larvae ($F_{7,74}=5.12$, $p=0.0018$). Mortality in the absolute controls was 2% for adults (Standard error of the mean, SEM=0.63), and 14.8% for larvae (SEM=2.47), values acceptable for this type of assay. The beetles, killed by the action of the fungus, showed progressive stiffness until complete mummification, with cadavers covered with white mycelia that later sporulated with powdery green conidia (Figure 4). Mortality data

was corrected, and the dose-mortality relationships were calculated (Table 3). A linear relationship between dose and mortality for both larvae and adults was found ($p > 0.05$). Mortality increased comparably with increased concentrations for both larvae and adults (Figure 3 and Table 3).

The LC_{50} was 9.4×10^6 and 1.8×10^4 conidia mL^{-1} for adults and larvae, respectively, and the LC_{90} was 1.6×10^9 and 9.2×10^6 conidia mL^{-1} for adults and larvae, respectively. Mortality increased in a comparable manner with increased concentrations for both larvae and adults (Adults: $p = 0.5$, $df = 3$, $\chi^2 = 2.13$ and Larvae: $p = 0.5$, $df = 3$, $\chi^2 = 2.35$).

Time-mortality test. The mean lethal time (LT_{50}) was estimated at a fungal concentration of 1×10^8 conidia mL^{-1} for both adults and larvae. The LT_{50} for adults was 10.1 days [95%, Confidence intervals (C.I.) 8.1-16.3] and was 4.3 days (95 % C.I. 3.8-4.7) for larvae.

Data available on the biological activity of *M. anisopliae* show great variability in activity with LC_{50} values between 1.7×10^3 conidia mL^{-1} and 1.4×10^7 conidia mL^{-1} (Quesada-Moraga *et al.*, 2004; Shi and Feng, 2009). Our findings are comparable with the values found for *Hoplia philanthus* control (LC_{50} : 2.5×10^4 - 4.0×10^6 conidia mL^{-1}) (Ansari *et al.*, 2004). *Eurygaster integriceps* (Hemiptera:

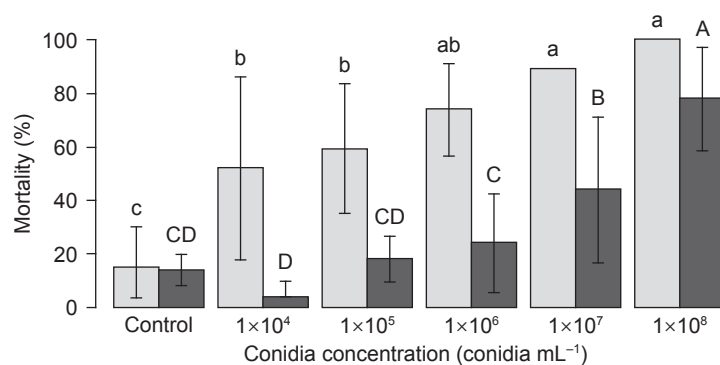


Figure 3. Mortality caused by *M. anisopliae* CPMa1502 on *D. neivai* larvae (□) and adults (■) under laboratory conditions. Values with different uppercase (adults) or lowercase (larvae) letters did not show statistical differences according to the LDS Test ($p < 0.05$).

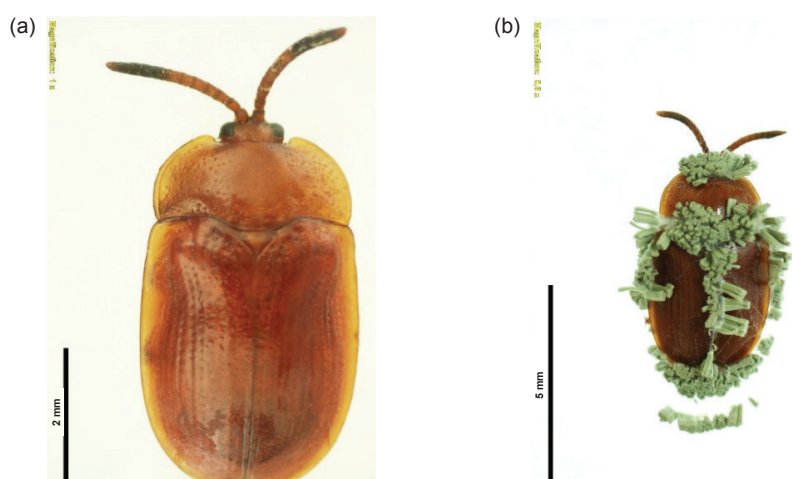


Figure 4. Typical symptoms of infected beetles by *M. anisopliae* CPMa1502: (a) healthy beetle from a control treatment; (b) fungi-infected beetle with mycelia formation and sporulation.

TABLE 3. ESTIMATION OF LC_{50} AND LC_{90} OF *M. anisopliae* CPMa1502 ON *D. neivai* ADULTS AND LARVAE UNDER LABORATORY CONDITIONS

Life stage	LC_{50} (conidia mL^{-1})	Confidence intervals (C.I.) (conidia mL^{-1})		LC_{90} (conidia mL^{-1})	Probit analysis			
		Lower	Upper		df	χ^2	p	Slope
Adults	9.4×10^6	4.5×10^6	2.3×10^7	1.6×10^9	3	2.1322	0.5454	0.576
Larvae	1.8×10^4	2.5×10^3	5.9×10^4	9.2×10^6	3	2.3540	0.4740	0.474

Note: df - degree of freedom; χ^2 - chi-square value; p - probability value.

Scutelleridae) nymphs were shown to be more susceptible than adults to different isolates and concentrations of *M. anisopliae*, with LT_{50} values between five and nine days for nymphs and 12 to 30 days for adults (Sedighi *et al.*, 2013). The diverse susceptibility of different life stages of insects to entomopathogenic fungi when they are used as pest control agents suggests that they should be applied when the insect is most susceptible to achieve the best efficacy, to curtail the spread of pests.

CONCLUSION

The growth parameters and biological characteristics of the CPMa1502 assessed in this study are fundamental to model its performance during further development as well as its fitness under real application conditions to control *D. neivai*. Results showed its high potential as an alternative for chemical pest control: Considerable conidia productivity on MAYP agar (1.1×10^9 conidia cm^{-2}), high tolerance to different growth conditions [pH (5-9) and temperature (below 30°C)], which will enable it to survive common conditions of oil palm plantations in Colombia. Conidia interaction with cuticle also suggests that CPMa1512 is a good candidate for further development (adherence: 112 conidia mm^{-2} ; hydrophobicity 72%). Lastly, the high susceptibility of *D. neivai* to CPMa1502 (Adults LC_{50} 9.4×10^6 conidia mL^{-1} ; Larvae LC_{50} 1.8×10^4 conidia mL^{-1}) show its insecticidal capacity and the potential for the development of the first biopesticide for this insect pest. This research provides the background needed to continue with the next steps of biopesticide development, standardising the fungal production, and formulation, and determining the biological activity of the formulated product under field conditions.

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FISH SPECIES RICHNESS, THEIR IMPORTANCE AND CONSERVATION STATUS IN TROPICAL OIL PALM AGROECOSYSTEM OF TERENGGANU, PENINSULAR MALAYSIA

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ABSTRACT

Oil palm cultivation is expanding especially in Malaysia, where natural land occupied by small streams and rivers has been converted into an agroecosystem. This study assessed and estimated fish species richness and fish diversity in the tropical oil palm plantation in Terengganu, Peninsular Malaysia, along with their importance, and conservation status. From 57 fish species recorded, 28 species were suitable as ornamentals, 21 species for both food and ornamentals, and four species were considered suitable as ornamentals, food, and sport fishing. About 54 fish species were classified in the Least Concern (LC) category while one species each was classified in the Data Deficient (DD) and Not Evaluated (NE) categories. More species can be recorded by increasing the sampling efforts as suggested by Chao 1 species richness estimator. Tributary Sungai Nerus has the highest fish species diversity and species richness, while Sungai Jeneris has the lowest fish species diversity, species richness, and species evenness. Continuous data collection can determine the true fish species richness and fish diversity in the oil palm plantation, which can help to suggest good management schemes to conserve fish fauna in this agroecosystem.

Keywords: agroecosystem, conservation, fish diversity, freshwater fish, sustainable.

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INTRODUCTION

The effect of land changes on organisms can vary; the species richness can decrease (Wilkinson *et al.*, 2018), increase (Lorion and Kennedy, 2009) or be unaltered (Bojsen and Barriga, 2002). Land use has been criticised due to the rapid expansion which had caused habitat destruction and subsequent loss of biodiversity (Immerzeel *et al.*, 2014). It had altered

the habitat structure of the rivers and streams (Ferreira *et al.*, 2018) through soil erosion and led to pesticide run-off into the water bodies. This will subsequently affect the aquatic organism in their microhabitat especially fish (Wilkinson *et al.*, 2018). The impact of land conversion is irreversible (Dislich *et al.*, 2017; Moreno-Mateos *et al.*, 2012) but can be reduced to sustain the survival of multi-diverse flora and fauna.

To our knowledge, a specific study on fish diversity in freshwater streams associated with oil palm monoculture plantations in Peninsular Malaysia has never been reported before. In the eastern part of Peninsular Malaysia, specifically in the state of Terengganu, most of the oil palm plantation areas reside in the adjacent forest/forest reserves and peatlands (Shevade and Loboda, 2019). In total, 171 548 ha of land in Terengganu were used for oil palm plantations until 2017 (Census and Economic Information Centre, 2020).

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Terengganu state has been chosen as the study site since Terengganu itself is very unique and ichthyodiverse, as 11 new species of freshwater fishes were previously described for the first time (type locality) from its river systems and water-bodies, namely *Brevibora cheeya*, *Rasbora notura*, *Pangio alcoides*, *Pangio filinaris*, *Pangio piperata*, *Homaloptera parclitella*, *Amblyceps foratum*, *Akysis hendricksonii*, *Nanobagrus lemniscatus*, *Betta stigmata*, and *Parosphromenus paludicola* (Kottelat, 2013). The list goes on with the description of the new species of other animals, such as crabs (Ng, 2020; Ng and Ahmad, 2016) and reptiles (Grismer *et al.*, 2018; Sumarli *et al.*, 2016). The trends show that there are chances of getting more species if more surveys were done in the selected area in Terengganu. Terengganu state also supported 103.67 t of inland fisheries landing worth USD554.81 (excluding dams) in 2019 (Department of Fisheries, 2020). The inland fisheries from the rivers in Terengganu are dominated by the catfish from the genus *Hemibagrus* sp., with the highest retail values (based on the total catches), followed by *Barbonymus schwanefeldii*, *Tor tambra*, and *Neolissochilus soroides* while other freshwater fish species were utilised at a smaller scale (Department of Fisheries, 2020).

In this study, we asked three research questions with regard to fish diversity in the oil palm plantation; 1) The total fish species richness and estimated fish species, 2) The conservation status and the importance of fish species, and 3) The significance of fish diversity among the streams and rivers. Despite having many small streams in the oil palm plantation, fish diversity and fish species richness in the streams within the plantations are rarely studied. It is partially unknown whether the fish community can fully adapt to such land modifications and frequent disturbances. This study

is crucial for the management and conservation of aquatic resources in the tropical agroecosystem, and for the community's livelihood which depends on freshwater fishes for food and economic enhancement.

MATERIALS AND METHODS

Study Areas

The study was carried out at nine selected streams in oil palm plantations in Terengganu. All streams are freshwater streams but Sg. Ular (Sg. = Sungai, means river) which is a blackwater stream (peatland) (Figure 1). The majority of the streams are dominated by sandy bottom substrate, with slow to moderate water flow. All the streams sampled in this study were approximately 4-5 m wide, and the water depth were below 1.0 m. All the streams, except Sg. Ular, resides in the catchment area of Sungai Terengganu, with a size of 4560 km² which spans across three districts; Kuala Terengganu, Hulu Terengganu, and Setiu (Department of Irrigation and Drainage, 2015). Fish data collection sites were chosen haphazardly, in the middle reach of the stream based on the site accessibility. The haphazard sampling method is commonly used by ecologists, where the sampling sites were chosen opportunistically depending on the habitats and time (Andrew and Mapstone, 1987), while the target habitat is either remote, poorly studied, or in the underwater environments (Smith *et al.*, 2017). The Sg. Ular blackwater stream originated from the peatlands area in Tasik Berombak, and flows through a peat swamp forest and oil palm plantations before being discharged into the lagoon of Setiu wetland.

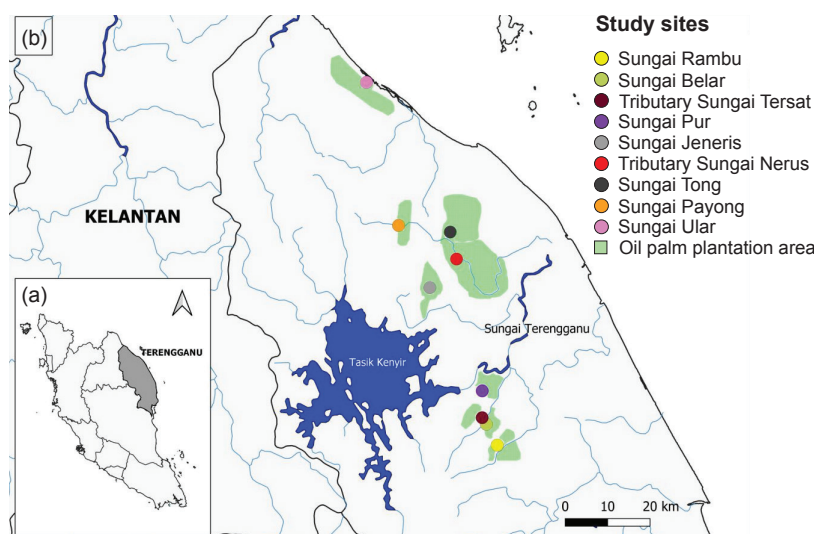


Figure 1. (a) The map of Terengganu located in the eastern part of Peninsular Malaysia, and (b) the study sites comprise of nine streams within the oil palm plantations in Terengganu.

Data Collections

Samplings were conducted from June 2018 to December 2018. The electrofishing technique was done using a backpack electrofisher model LR-24 (Smith Roots Inc.) by wading in an upstream direction. There were three plots (every 30 m interval for each plot), sampled along 90 m reach of each stream. The distance for the sampling plots was determined as suggested by Patton *et al.* (2000) and Dauwalter and Pert (2003), for a minimum of 50 m length. The seine net was handled by two people; holding at the edge of both sides of the seine net to form a U-shape, to block fishes which drifted in the fast-flowing water current. Long-handled scoop nets were used to retain and catch the stunned fish. Fishes caught in the seine net were carefully removed and placed in a plastic basket immersed in flowing water, to reduce fish mortality. We used the electrofishing techniques in this study to maximise the sampling efforts, as it is more effective than the single hand-seining methods in tropical streams (Deacon *et al.*, 2017).

Fish Identification and Preservation

The fishes were first euthanised in tricaine methanesulphonate (MS-222) before being identified *in situ*. The voucher specimens (one to three individuals for each species) were taken for further confirmation of the species' identity. The fishes were identified according to the taxonomic keys following Zakaria-Ismail *et al.* (2019) and verified based on Fricke *et al.* (2022). The specimens were preserved in 10% formalin for at least two weeks. The specimens were then immersed in tap water to remove the fixative and preserved in 70% of ethanol solution for long-term preservation. The preserved fishes are kept in the General Biology Laboratory of Universiti Malaysia Terengganu Zoological Collections (UMTZC). The IUCN Status for all fish species identified were based on The IUCN Red List of Threatened Species version 2021-3, while the guidelines for "Uses" were referred primarily based on The IUCN Red List of Threatened Species version 2021-3, under General Use and Trade Classification Scheme (Version 1.0).

Statistical Analysis

Species accumulation curves (SAC) were plotted into two types of curves (abundance-based; the total number of individuals and sample-based; the number of streams) to determine the sampling completeness. The species richness estimator, Chao 1 was also employed to compare the observed species richness (S_{obs}) and estimated species richness (S_{est}) for all streams. There were six diversity indices

used to measure fish diversity namely Shannon (H) and Simpson (1-D) for species diversity; Margalef (R1) and Menhinick (R2) for species richness; Equitability (EH) and Evenness (E) for species evenness. A diversity t-test was performed via a pairwise comparison between all streams to determine the significant difference in diversity, based on Shannon (H) and Simpson (1-D) values. We chose both indices as it measures the diversity based on two main components of species diversity; species richness and species evenness (Gorelick, 2006), while the other indices used in this study only measure species richness (R1 and R2), and species evenness (EH and E) (Magurran, 2004). All the statistical analyses were done using Paleontological Statistics Software (PAST version 4.03) (Hammer *et al.*, 2001).

RESULTS AND DISCUSSION

A total of 57 species of freshwater fish species from 20 families was recorded from nine streams in oil palm plantations in Terengganu, Peninsular Malaysia (Table 1). The family Cyprinidae was the most dominant family with the highest species richness (15 species) followed by the family Danionidae, Balitoridae, and Bagridae with five species respectively. The family Cobitidae has four species, followed by Channidae (three species), while the families Nemacheilidae, Akysidae, Zenarchopteridae, Mastacembelidae, Gobiidae, Osphronemidae has two species. Other families were represented by single species only. Among all streams, Sg. Tong has the highest number of species (18 species), followed by Sg. Pur, Sg. Payong and Tributary Sg. Nerus (17 species each), Sg. Rambu (16 species), Sg. Tersat (15 species), Sg. Ular (13 species), Sg. Belar (11 species) and Sg. Jeneris (9 species) (Table 1; Figure 2). Based on The IUCN Red List of Threatened Species version 2021-3, the majority of the species was in the Least Concern (LC) categories (96%; 54 species), followed by Not Evaluated (NE) (2%; 1 species), Data Deficient (DD) (2%; 1 species). One species was not assessed as it could be an undescribed species (*Rasbora cf. notura*). About 28 fish species were regarded as ornamental fishes (i.e., *Nemacheilus masyae*, *Homaloptera parclitella*, and *Rasbora paucisqualis*), while 21 species were utilised for both food and ornamental purposes (i.e., *Acantopsis dialuzona*, *Ceratogarra cambodgiensis*, *Mastacembelus favus*). Three species were used as food only (*Hemibagrus capitulum*, *H. gracilis*, and *Monopterus javanensis*), one species was used as food and for sport fishing (*Channa striata*), while four species have all three importance as food, ornamental, and for sport fishing (*Channa lucius*, *C. melasoma*, *Hampala macrolepidota*, and *Neolissochilus soroides*) (Table 1).

TABLE 1. FISH SPECIES LIST RECORDED IN OIL PALM STREAMS IN TERENGGANU, PENINSULAR MALAYSIA

Family and species	Sg. Rambu	Sg. Belar	Tributary Sg. Tersat	Sg. Pur	Sg. Jeneris	Tributary Sg. Nerus	Sg. Tong	Sg. Payong	Sg. Ular	IUCN status (2021-3)	Uses
Notopteridae											
<i>Notopterus notopterus</i>	-	-	-	-	-	-	-	-	+	LC	F, O
Cobitidae											
<i>Acantopsis dialuzona</i>	-	+	-	+	+	+	+	-	-	LC	F, O
<i>Acanthopsoides molobrion</i>	-	-	-	+	+	-	+	-	-	LC	O
<i>Pangio doriae</i>	-	-	-	-	-	+	-	-	-	LC	O
<i>Pangio semicineta</i>	-	-	-	-	-	-	+	-	-	LC	O
Balitoridae											
<i>Balitoropsis zollingeri</i>	+	+	-	-	-	+	-	+	-	LC	O
<i>Homaloptera ogilviei</i>	-	+	-	-	-	-	-	+	-	LC	O
<i>Homaloptera parclitella</i>	-	-	-	+	-	-	-	+	-	LC	O
<i>Homalopteroides nebulosus</i>	-	-	-	+	-	+	+	-	-	LC	O
<i>Homalopteroides tweediei</i>	+	-	-	-	-	-	-	-	-	LC	O
Nemacheilidae											
<i>Nemacheilus masyae</i>	-	+	-	-	+	+	+	-	-	LC	O
<i>Nemacheilus selangoricus</i>	-	-	+	-	-	-	-	-	-	LC	O
Cyprinidae											
<i>Barbodes sellifer</i>	+	+	+	+	-	+	-	+	-	LC	F, O
<i>Barbodes cf. binotatus</i>	-	-	-	-	-	-	-	-	+	LC	F, O
<i>Barbodes lateristriga</i>	+	-	+	+	-	-	-	-	-	LC	F, O
<i>Crossocheilus oblongus</i>	-	-	-	-	-	-	-	+	-	LC	O
<i>Cyclocheilichthys apogon</i>	-	-	+	-	-	-	-	-	-	LC	F
<i>Ceratogarra cambodgiensis</i>	+	-	-	-	-	+	-	+	-	LC	F, O
<i>Hampala macrolepidota</i>	-	-	-	-	-	-	+	-	-	LC	F, S
<i>Labiobarbus leptocheilus</i>	-	-	-	-	-	-	+	+	-	LC	F
<i>Lobocheilos rhabdoura</i>	-	-	-	-	-	-	-	+	-	LC	F
<i>Mystacoleucus obtusirostris</i>	-	-	-	+	-	+	-	-	-	LC	F, O
<i>Neolissochilus soroides</i>	+	-	-	-	-	-	-	-	-	LC	F, S
<i>Osteochilus vittatus</i>	-	+	-	-	-	-	+	+	+	LC	F, O
<i>Osteochilus waandersii</i>	+	+	-	-	-	-	+	+	-	LC	F, O
<i>Poropuntius normani</i>	+	-	-	-	-	+	-	-	-	LC	F, O
<i>Puntigrus partipentazona</i>	-	-	-	-	-	-	-	-	+	LC	O

TABLE 1. FISH SPECIES LIST RECORDED IN OIL PALM STREAMS IN TERENGGANU, PENINSULAR MALAYSIA (continued)

Family and species	Sg. Rambu	Sg. Belar	Tributary Sg. Tersat	Sg. Pur	Sg. Jeneris	Tributary Sg. Nerus	Sg. Tong	Sg. Payong	Sg. Ular	IUCN status (2021-3)	Uses
Danionidae											
<i>Rasbora cf. notura</i>	-	-	+	+	-	-	-	-	-	-	-
<i>Rasbora myersi</i>	+	-	+	-	+	-	+	+	-	NE	O
<i>Rasbora notura</i>	+	+	+	+	-	+	+	-	-	LC	O
<i>Rasbora paucisqualis</i>	-	-	-	+	-	-	-	-	-	LC	O
<i>Rasbora paviana</i>	-	-	-	-	-	-	-	-	+	LC	O
Bagridae											
<i>Hemibagrus capitulum</i>	-	+	+	-	-	-	+	-	+	LC	F
<i>Hemibagrus gracilis</i>	+	-	-	-	-	+	-	-	-	LC	F
<i>Leiocassis poeciloptera</i>	+	-	-	-	+	+	-	+	-	LC	O
<i>Mystus castaneus</i>	-	-	+	-	-	-	-	-	-	LC	F, O
<i>Pseudomystus stenomus</i>	-	-	-	-	+	-	-	-	-	LC	O
Akysidae											
<i>Acrochordonichthys rugosus</i>	-	-	-	-	+	-	-	-	-	LC	O
<i>Akysis hendricksoni</i>	-	-	-	-	+	-	-	-	-	LC	O
Amblycipitidae											
<i>Amblyceps foratum</i>	+	-	-	-	-	+	-	-	-	LC	O
Sisoridae											
<i>Glyptothorax fuscus</i>	+	+	+	+	+	+	-	+	-	LC	O
Siluridae											
<i>Silurichthys schneideri</i>	-	-	-	-	-	-	-	+	-	LC	F, O
Syngnathidae											
<i>Doryichthys martensii</i>	-	-	+	+	-	+	-	+	-	DD	O
Gobiidae											
<i>Glossogobius giuris</i>	-	-	-	-	-	-	-	-	+	LC	F, O
<i>Pseudogobiopsis oligactis</i>	-	-	-	-	-	-	-	-	+	LC	O
Mastacembelidae											
<i>Macrognathus circumcinctus</i>	+	-	+	+	-	+	-	+	-	LC	F, O
<i>Mastacembelus favus</i>	-	-	-	+	-	-	-	+	-	LC	F, O
Synbranchidae											
<i>Monopterus javanensis</i>	-	+	+	-	-	-	+	-	-	LC	F

TABLE 1. FISH SPECIES LIST RECORDED IN OIL PALM STREAMS IN TERENGGANU, PENINSULAR MALAYSIA (continued)

Family and species	Sg. Rambu	Sg. Belar	Tributary Sg. Tersat	Sg. Pur	Sg. Jeneris	Tributary Sg. Nerus	Sg. Tong	Sg. Payong	Sg. Ular	IUCN status (2021-3)	Uses
Osphronemidae											
<i>Trichopodus trichopterus</i>	-	-	-	-	-	-	+	-	-	LC	F, O
<i>Trichopsis vittata</i>	-	-	-	-	-	-	+	-	+	LC	O
Channidae											
<i>Channa lucius</i>	+	-	+	+	-	-	-	-	+	LC	F, O, S
<i>Channa melasoma</i>	-	-	-	+	-	-	-	-	-	LC	F, O, S
<i>Channa striata</i>	-	-	-	-	-	-	+	-	+	LC	F, S
Pristolepididae											
<i>Pristolepis grootii</i>	-	-	-	-	-	-	+	-	+	LC	F, O
Aplocheilidae											
<i>Aplocheilus armatus</i>	-	-	-	-	-	-	-	-	+	LC	O
Zenarchopteridae											
<i>Dermogenys collettei</i>	-	-	-	-	-	-	+	-	-	LC	O
<i>Hemirhamphodon pogonognathus</i>	-	-	+	+	-	+	-	-	-	LC	O
Total species	16	11	15	17	9	17	18	17	13		

Note: The IUCN status abbreviations are as follows: LC - least concern; DD - data deficient; NE - not evaluated.

F - food; O - ornamental; S - sport fishing; '+' - present; '-' - absence.

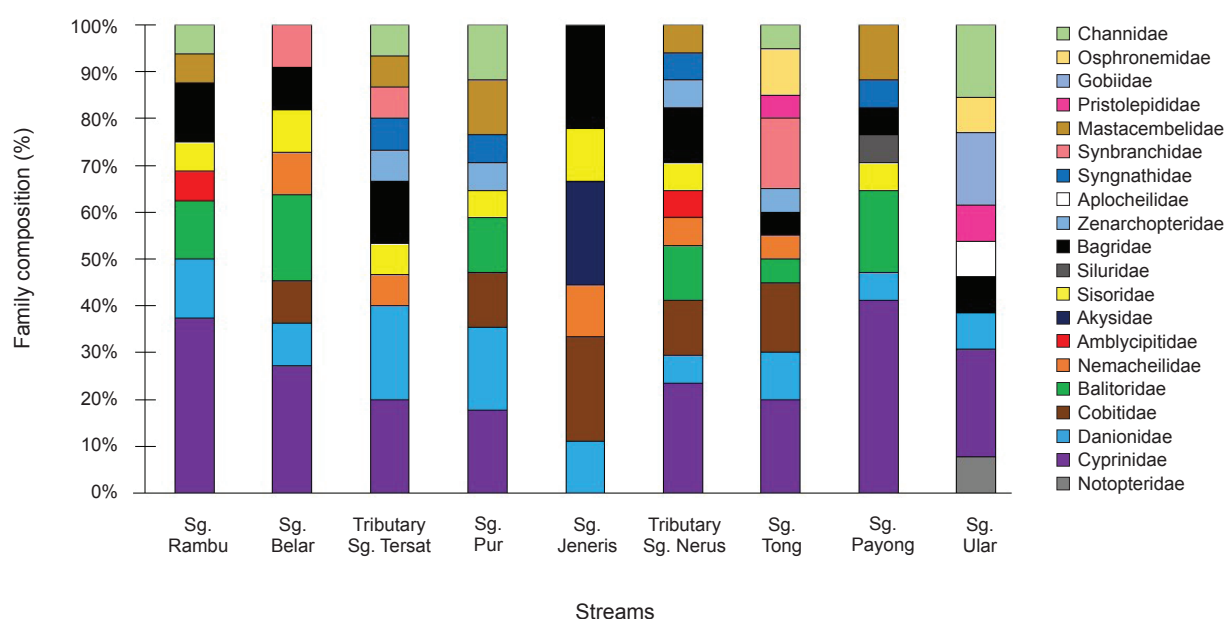


Figure 2. Family composition of freshwater fishes in nine streams in the oil palm plantation areas in Terengganu.

The species accumulation curves for both total abundance and stream numbers show no sign of reaching asymptotes based on the current sampling efforts (*Figures 3a and 3b*). The curves were steadily increasing, indicating that the sampling is not completed yet and more species may be added with the addition of sampling efforts (number of individuals and the number of streams). Plus, the species richness estimator, Chao 1 estimated more species than the observed species in each stream (*Figure 3c*).

The tributary of Sg. Nerus has shown the highest diversity indices value among all the streams except for Equitability and Evenness values, in which Sg. Belar has the highest Equitability and Evenness indices value, meanwhile Sg. Jeneris has shown the lowest diversity values for all indices among all the streams (*Table 2*). The diversity comparison showed no regular patterns in the significant difference of Shannon and Simpson values, except for Tributary Sg. Nerus and Sg. Jeneris, has a significant difference in Shannon and Simpson values with the other eight streams (*Table 3*).

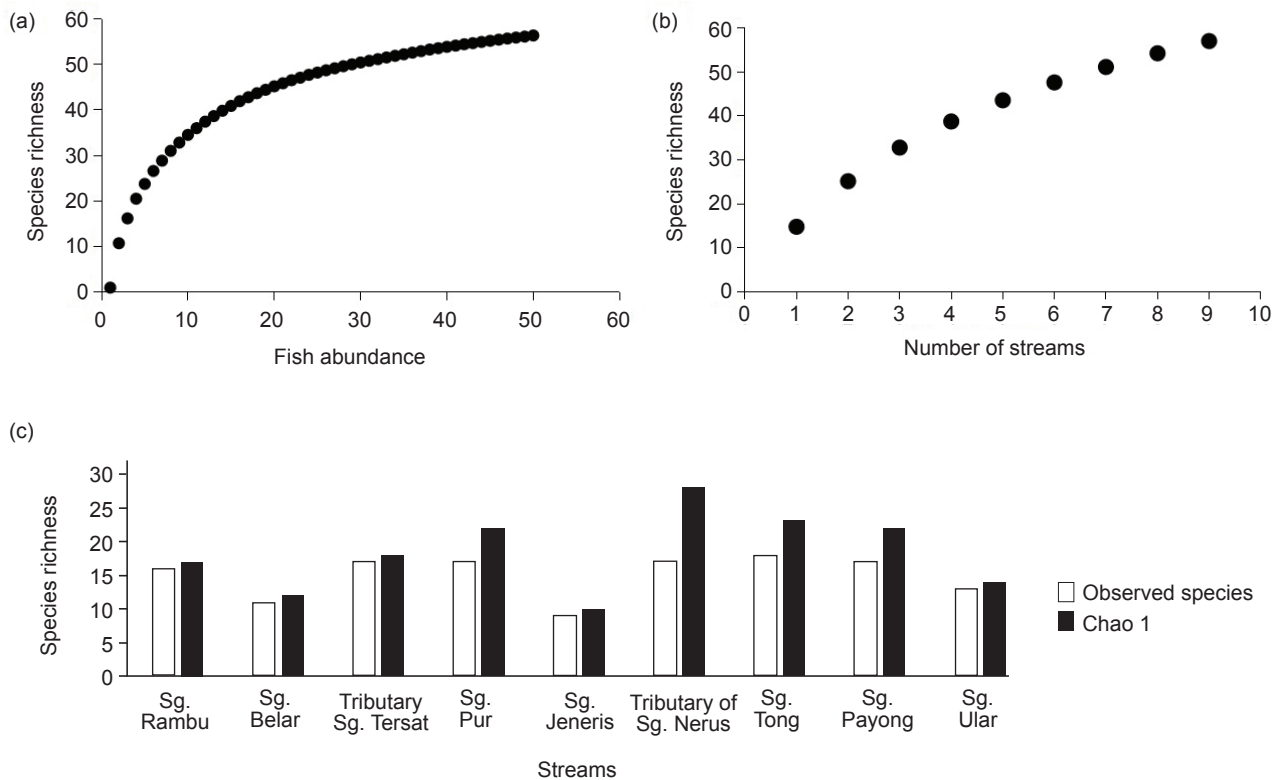


Figure 3. (a) Species accumulation curves for abundance and, (b) sample-based. (c) The estimated species richness by the estimator, Chao 1 also included for all streams.

TABLE 2. THE DIVERSITY VALUES IN NINE STREAMS IN THE OIL PALM PLANTATIONS

	Sg. Rambu	Sg. Belar	T. Sg. Nerus	Sg. Jeneris	Sg. Tong	Sg. Payong	Sg. Ular	T. Sg. Tersat	Sg. Pur
Shannon	2.031	2.167	**2.511	*1.378	2.163	2.344	2.054	2.253	2.307
Simpson	0.794	0.868	**0.901	*0.638	0.823	0.848	0.821	0.862	0.864
Margalef	3.067	2.659	**3.877	*1.903	3.842	4.156	2.955	3.213	3.662
Menhinik	1.387	1.677	**2.159	*1.100	1.567	2.480	1.707	1.698	1.913
Equitability	0.732	**0.904	0.886	*0.627	0.748	0.827	0.801	0.832	0.814
Evenness	0.476	**0.794	0.725	*0.441	0.483	0.6132	0.600	0.634	0.591

Note: '**' - the lowest diversity values while '***' - the highest diversity values. (Sg. = Sungai, T = Tributary).

TABLE 3. THE DIVERSITY COMPARISON BETWEEN NINE STREAMS USING SHANNON VALUES (H') AND SIMPSON VALUES (1-D)

		Shannon values (H')								
Streams		Sg. Rambu	Sg. Belar	T. Sg. Nerus	Sg. Jeneris	Sg. Tong	Sg. Payong	Sg. Ular	T. Sg. Tersat	Sg. Pur
Simpson Index (1-D)	Sg. Rambu			Sig	Sig					
	Sg. Belar				Sig					
	T. Sg. Nerus	Sig			Sig			Sig		
	Sg. Jeneris	Sig	Sig	Sig		Sig	Sig	Sig	Sig	Sig
	Sg. Tong			Sig	Sig					
	Sg. Payong			Sig	Sig					
	Sg. Ular		Sig	Sig	Sig					
	T. Sg. Tersat	Sig			Sig	Sig		Sig		
	Sg. Pur	Sig		Sig	Sig	Sig		Sig		

Note: Only significant different comparisons were shown to increase the clarity of the table (Sg. = Sungai, T = Tributary).

The current findings show that the family Cyprinidae and Danionidae make up the major family composition in this agroecosystem. This result is akin to the other studies on the agricultural landscape such as rice fields (Aqmal-Naser and Ahmad, 2018a; 2018b) and other plantation-related habitats (Ahmad *et al.*, 2018; Aqmal-Naser and Ahmad, 2021). It is historically well-known that Cyprinidae is the dominant family in the freshwater ecosystems in Peninsular Malaysia (Zakaria-Ismail *et al.*, 2019), hence its dominance is not surprising. The total number of species richness, however, contributed to a small portion (19.72%) of the total 289 species of freshwater fishes recorded in Peninsular Malaysia (Zakaria-Ismail *et al.*, 2019).

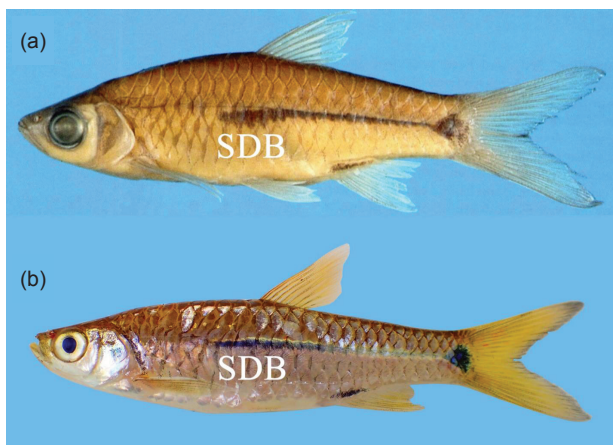
Almost all of the species were in the LC category and did not face any immediate threats, widespread (for certain species) and abundant in other places. The absence of rules and regulations regarding the over-exploitation of fish from wild sources, becomes a concern, especially for the fishes with higher ornamental values. More strict rules and regulations need to be implemented in Malaysia to avoid the over-collection of ornamental fish. The Brazilian government has introduced a ban on the export of the highly-sought fish species of the genus *Hypancistrus* (Isbrücker and Nijssen, 1991) to sustain the long-term sustainability of wild-capture fisheries (Pedersen, 2016). In addition, one species, *Hypancistrus zebra* has been prohibited to be collected, transported or traded in Brazil's aquarium fish trade as it was placed on the Brazilian Red List of Endangered species in 2004 (Evers *et al.*, 2019). In Peninsular Malaysia, more studies are needed on the biology and ecology aspects of its fish species (*i.e.*, for species in the DD and NE categories) for proper long-term management of fisheries resources.

Species diversity, species richness and species evenness indices values for the fishes among the streams varied but the tributary of Sg. Nerus shows the highest diversity values but with low evenness. The species richness in the river is higher but the number of individuals (abundance) for each species is not uniform and shows dominance by certain species. Meanwhile, Sg. Belar which shows the highest evenness value indicated that fish abundance for each species in the river can be considered uniform. Sg. Jeneris shows the lower diversity values but the highest evenness values, which means that the species richness was the lowest but with an approximately uniform number of individuals for each species. Besides, Sg. Jeneris also show a significant difference in species diversity to the lowest number of species richness compared to other streams. The result is aligned with the estimated number of species richness for a tributary of Sg. Nerus, which suggested more species could be recorded with additional sampling efforts (Figure 3c). Fish diversity was affected by various types of disturbances including habitat fragmentation (Poulet, 2007), habitat alteration (Holcik, 2003) and the expansion of agriculture that led to the absence of riparian zone (Wilkinson *et al.*, 2018). The repetitive sampling approach (three times sampling visits) was recommended for more confident detection of the species richness of fishes in the streams (Reid and Haxton, 2017). Furthermore, future studies should consider the effect of longitudinal profiles of the stream corridor (upper stream, middle stream, and downstream), and the intensity of oil palm coverage toward fish diversity.

The ornamental fish sector in Malaysia has a prodigious market value. The sector is worth USD125 451 520.82 (RM506 447 789.55) in 2019, while Terengganu contributes about USD388 865.20

(RM1 569 848.80) to the ornamental fish values in Malaysia (Department of Fisheries, 2020). However, the practice of aquarium dumping especially from the ornamental fish species has allowed the establishment of invasive alien species in the natural water bodies (Aqmal-Naser and Ahmad, 2020a). Luckily, no alien fish species were recorded in the study sites, suggesting that the habitat may not be suitable for alien species, or not yet been invaded. Precaution steps are needed by monitoring the presence of alien species before it is too late for eradication.

One small-sized species was not assessed and was identified as *Rasbora cf. notura*. The specimen resembles the holotype of *Rasbora notura* described by Kottelat (2005) but differs by its subdorsal blotch (SDB) and snout (Figure 4). Both *Rasboras* have similar general morphologies and a diamond-shaped blotch at the caudal peduncles. However, the real *R. notura* has a wider anterior subdorsal portion than the posterior part (Lumbantobing, 2014), while the second example has tapered and almost the same thickness of SDB, thus it does not fit into the description by Kottelat (2005) and Lumbantobing (2014) for the characteristic of *R. notura*. In addition, the snout of *R. notura* is more pointed compared to the snout of *Rasbora cf. notura* in this study, which is shorter and blunt (Figure 4). Currently, we regarded this species as another form of *R. notura* species, and it may be a complex which needs further taxonomic study.



Note: SDB - subdorsal blotch.

Figure 4. (a) The holotype of *Rasbora notura* from Kottelat (2005), and (b) *Rasbora cf. notura* from this study.

The species accumulation curves and the estimation of Chao 1 demonstrated that there should be more species to be recorded with the additional sampling efforts. The sampling efforts in terms of the number of individuals were the most appropriate measure suggested because it helps to avoid bias (Moreno and Halffter, 2001; Willott, 2001). We employed the electrofishing techniques in this

study to maximise the sampling efforts, as the use of the method was proven to be more effective than the single hand-seining methods, thus increasing the chances of getting higher abundance and a higher diversity of fishes in the tropical streams (Deacon *et al.*, 2017). In another study, Almeida *et al.* (2017) suggested that the species richness can be reasonably estimated if the transect length was 10 times the stream width or less. Several studies also proposed a transect length of 150 m (Meador *et al.*, 2003) or a minimum of 50 m length to collect 90% of the species in the streams (Dauwalter and Pert, 2003; Patton *et al.*, 2000). The suggested transect length also was corresponding to our transect length which was 90 m (for 3 plots). Future works on the fish inventories should be focusing on repetitive sampling, rather than single inventories, for documenting fish species richness in the tropical streams in Malaysia.

The species richness in total is relatively higher for a single data collection, (at every 30 m intervals along a 90 m reach), hence causing a relatively lower average species richness per stream (less than 15 species), compared to the earlier studies in the plantation areas (Ahmad *et al.*, 2018; Aqmal-Naser and Ahmad, 2021). In terms of conservation, the fragmented habitat (monotonous habitat) within the streams in the oil palm plantations can affect the fish composition and beta diversity, especially the habitat-specialised fish. The rheophilic species depend on the gradient and high-water velocity for reproduction and development (Birnie-Gauvin *et al.*, 2017). The rheophilic species in this area live in the fast-flowing and rapid water current and might need to migrate to the upper stream or another pristine habitat as the increase in sediment loading may cause habitat deterioration, which is not suitable for the fish. The heavy sediment loading in the streams associated with logging had shown a smaller number of fish species, and the absence of many rheophilic fish species compared to the moderately disturbed streams (Aqmal-Naser *et al.*, 2020b).

The riparian reserves for the oil palm plantations are required by the law of the Malaysian Government in 1965 and mandatory for the participants of the Roundtable on Sustainable Palm Oil (RSPO) certification program (Giam *et al.*, 2015). In addition, Malaysia also has the Malaysian Sustainable Palm Oil (MSPO) certification scheme which aims to establish a sustainable palm oil operation. It is also parallel to the fifth principle of RSPO, which is to ensure the conservation of biodiversity, natural resources, and the ecosystem by reducing pollution. It is crucial to have a riparian buffer to decrease the impact of disturbance on the aquatic ecosystems. The riparian buffer had successfully maintained fish species richness, functional diversity and biomass in the streams (Giam *et al.*, 2015) and fish abundance (Deere *et al.*, 2021) compared to the no riparian buffer zone in the oil palm monoculture of

Southeast Asia. Most of the streams sampled in this study did not have riparian reserves, which might explain the lower species richness recorded. Both RSPO and MSPO programs are known to guide the farmers with sustainability standards but whether they can maintain the aquatic biodiversity, remains a challenge with regard to law enforcement of the riparian reserves. Even though the species richness was considered lower, the estimated species richness still showed increasing trends and could indicate an altered habitat but with natural cycles which would possibly enable the fish diversity to be sustained. However, this requires continuous monitoring to validate the hypothesis.

CONCLUSION

Our study had shown that fish species richness in the oil palm plantations has two major importance; as ornamental and as food. Continuous inventories and monitoring are the keys to recording all known fish species inhabiting the oil palm agroecosystem. However, most of the species recorded at present are not very essential for fish species conservation. They bring some value to the local people and should be a priority in future planning regarding the expansion of oil palm plantations. The non-compliance of farmers on the riparian reserves will become the major factor that contributes to the reduction of fish diversity in this agroecosystem. It is necessary to continue protecting the primary forest reserve, and creating a riparian forest in the already established oil palm plantations, while at the same time, steering the possible win-win situations for both ecosystem function and human livelihood.

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ENDOGENOUS HORMONES FROM THE YOUNG LEAVES OF OIL PALM (*Elaeis guineensis* Jacq.): A RESPONSE TO *In Vitro* CALLUS AND EMBRYOID INDUCTION

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ABSTRACT

Oil palm is a plant that produces vegetable oil. Quality oil palm seedlings with superior characteristics can be produced clonally using tissue culture techniques. The selection of explant sources is very important, and one of the explant sources that can be used is the young oil palm leaves. The ability of callogenesis and embryogenesis remains low, and this is thought to be related to endogenous hormones. This study is aimed to identify the types of endogenous hormones in explants of young clonal oil palm leaves and their relationship to callus and embryoid induction. The leaf midribs of six young oil palms were selected as explant sources. Explants were extracted and analysed for endogenous hormone content using high-performance liquid chromatography. The results of the analysis obtained various types of endogenous hormones in four oil palm clones. Zeatin hormones, ABA and IBA were found in two clones. Nine hormones were found in the other two clones, namely zeatin, kinetin, gibberellic acid, IAA, BAP, ABA, IBA, 2,4-dichlorophenoxyacetic acid, and naphthalene acetic acid with various concentrations. Young oil palm leaves with a more complete content of endogenous hormones have the potential to produce embryogenic callus in greater numbers and can produce embryoids to ensure that they can support the production of plantlets using tissue culture technology.

Keywords: ABA, benzyl amino purine (BAP), embryogenic callus, zeatin, 2,4-D.

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INTRODUCTION

Oil palm is an economically important plant, with almost all plant's parts able to be utilised,

most importantly its oil can be processed into various food and non-food products. The oil palm fruit consists of a pulp (mesocarp) and a kernel which contains about 45%-50% (Owoyele and Owolobi, 2014) and 47% of oil (Naher *et al.*, 2013), respectively. Compared with other oil-producing plant commodities such as soybean, sunflower, canola and corn, the oil palm remains as a prime commodity due to its much higher oil productivity per planting area (Naher *et al.*, 2013).

The tissue culture technique has been introduced in oil palm plant-related activities since 1970 to meet the demand for superior seedlings (Ismail *et al.*, 2010; Staritsky, 1970). Oil palm plant has only one growing point (single apical meristem) which makes it difficult to propagate vegetatively in the field, where tissue culture technology offers a solution (Mariani *et al.*, 2014). Oil palm tissue culture requires explants sources that can be derived

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from young leaves, roots, flowers (inflorescence), and embryos (Weckx *et al.*, 2019), each having its advantages and disadvantages. Young leaves are mostly selected as the source of explants for oil palm tissue culture because of their availability in large quantities. One ortet can produce hundreds or even thousands of explant pieces with relatively higher sterility (Setiowati *et al.*, 2013).

The tissue culture technique has been applied to generate quality oil palm seedlings with increased crude palm oil productivity (Weckx *et al.*, 2019). The resulting clonal seedlings possess quality characteristics identical to their parents and can be produced without season constraints (Soh *et al.*, 2011). The *in vitro* clonal propagation of oil palm, however, still faces the problems of poor productivity of callus and somatic embryo, with the callogenesis and embryogenesis rates as low as 14.00%-19.00% and 3.00%-7.00% (Kushairi *et al.*, 2010), 30.00% and 5.00% (Alwee *et al.*, 2010), 10.80% and 3.00% (Yusnita and Hapsoro, 2011), 33.33% and 7.69% (Sanputawong and Te-Chato, 2012), 15.00% and 3.00% (Marbun *et al.*, 2015), 1.39%-30.56% and 3.63% (Wiendi *et al.*, 2015), 13.10%-22.30% and 5.00% (Gomes *et al.*, 2017), as well as 20.48% and 3.00%-5.00% (Karyanti *et al.*, 2019), respectively. The use of broodstock sources with different genotypes is thought to contain different endogenous hormones and affect callus and embryoid induction abilities (Elhiti *et al.*, 2013).

The quality of the callus formed in oil palm tissue culture is another crucial issue since its quality affects the ability to form embryoids (or embryonic calli) which are the precursors of the plantlets to be produced. Callus formation is induced by the interplay of both exogenous and endogenous hormones. The use of quantifiable hormone concentrations can suppress the occurrence of somaclonal variations, especially when young leaf explants are used (Bairu *et al.*, 2011). The administration of measurable synthetic hormones was unable to increase the number and quality of the callus formed, triggering speculation that the problem lay in the unidentified endogenous hormones present in the source explants. These auxin and cytokinin types of endogenous hormones could affect the callus and embryoid formation (Xue *et al.*, 2020). Thus, this study aims to measure the content of endogenous hormones in the young leaves of oil palm plants and to determine their effect on *in vitro* callus and embryoid induction.

MATERIALS AND METHODS

This study was carried out at the Biotechnology Laboratory, National Research and Innovation

Agency, Science and Technology Park, South Tangerang City, Banten, Indonesia. The activities consisted of *in vitro* plant tissue culture in the Tissue Culture Laboratory and hormone measurement in the Laboratory of Analytical Chemistry.

Explant Source for Callus Induction

Young leaves were sampled from four oil palm trees (broodstocks) of different genotypes which had been determined beforehand, based on normal morphological data and high production. The selected broodstock was the result of *Dura* Deli × *Pisifera* Nigeria Origin, hereinafter referred to as a clone. Sampling was conducted in 2018 from oil palm plantations located in Kumai, Central Kalimantan. All four oil palm trees used as a source of explants were about 7 years old and denoted as clone-1, clone-2, clone-3, and clone-4 (Figure 1a, b, c and d). The shoots of each clone were carefully cut to avoid damaging the growing point and were brought to the laboratory (Figure 1e).

The young shoots were cleaned and carefully opened, exposing the midrib layers (Figure 1f). The six youngest leaf midribs commonly used in oil palm tissue culture labelled as midrib layer numbers 5, 6, 7, 8, 9 and 10 (Figure 1g), were used as the source of explants for callus induction and as sample for analysis of endogenous hormone content (Figure 1h). Each midrib was cut into pieces of about 1.5 cm (Figure 1i). The young leaf pieces to be used as explant for callus induction were sterilised (Figure 1j), and then planted on a callus induction medium (Figure 1k).

Explant Source for High-Performance Liquid Chromatography (HPLC) Analysis of Endogenous Hormones

A method similar to the one for preparing the explants for callus induction was used, but with no sterilisation. The explant sample was weighed out of 100 g (Figure 1j) and extracted before analysis using HPLC. Each sample was replicated three times.

Endogenous Hormone Analysis

The hormone was analysed using the HPLC method as described by Li *et al.* (2016) with modification. The explant samples were macerated using ethyl acetate (Emsure) and then homogenised using a magnetic stirrer for approximately 24 hr. The macerated samples were filtered (Whatman 45 mm), and the filtrate obtained was centrifuged for 10 min at 3000 rpm. The centrifuged filtrate was separated from the solid and evaporated (Heidolph Laborota 4000 Rotary Evaporator) to dry, to derive endogenous

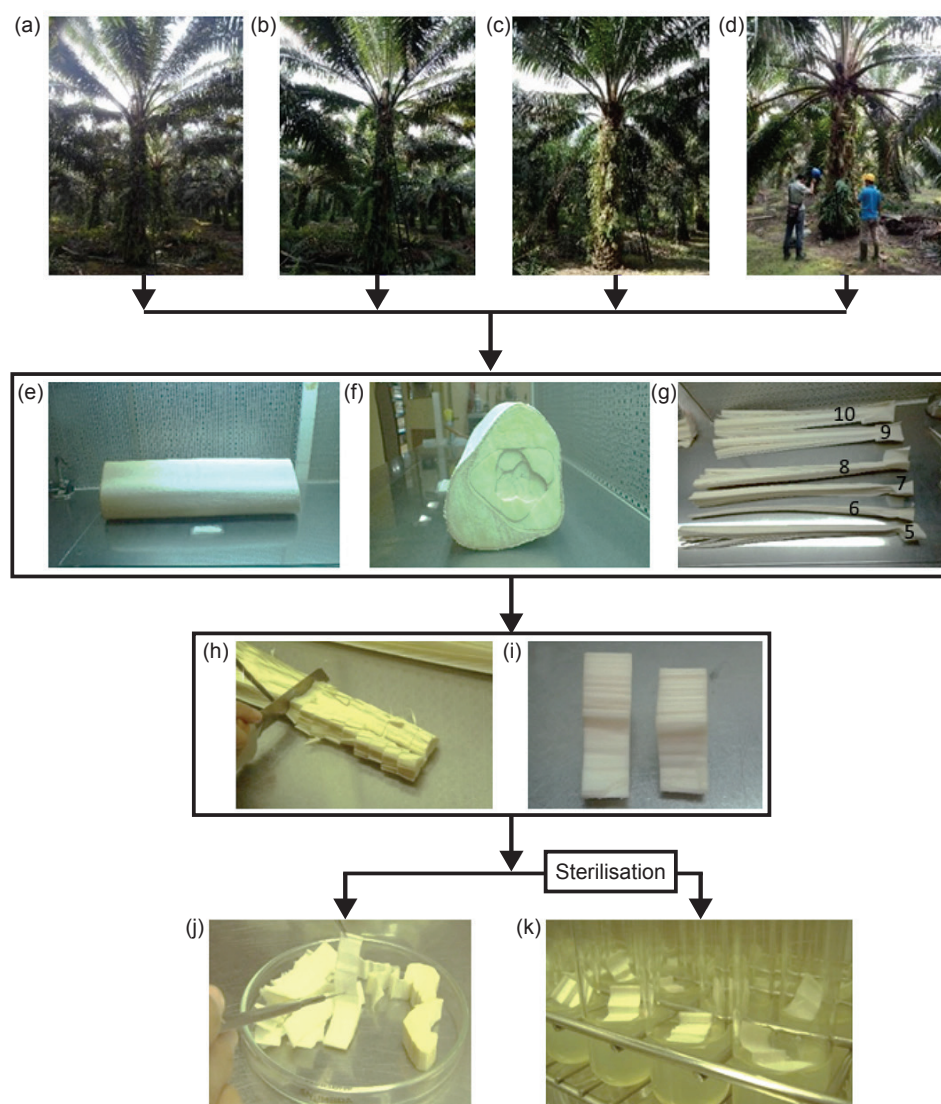


Figure 1. Source of explants for callus induction and endogenous hormone analysis: (a) clone-1, (b) clone-2, (c) clone-3, (d) clone-4, (e) shoots sampled from each clone, (f) cross-sectional area of shoots after cutting, (g) six midribs selected and numbered 5, 6, 7, 8, 9, and 10, (h) each midrib cut into pieces, (i) explant sized to 1.5 cm pieces, (j) leaf explants for endogenous hormone analysis, and (k) sterilised young leaf explants planted in callus induction media.

growth hormone extract which was subsequently dissolved in 5 mL of methanol (Merck). This methanolic solution was then concentrated again using a vacuum concentrator (Sakuma Ec-2000) for about 3 hr to evaporate the methanol. The concentrated extract was then dissolved again in 500 μ L methanol and later centrifuged at 1200 rpm for 10 min to separate and remove any remaining precipitate from the hormone extract solution sample, to be assayed using an HPLC (Hitachi L-2130).

HPLC analysis was performed by injecting the extract solution sample (10 μ L). The mobile phase used was methanol:formic acid 0.1% pH 3.2 with a ratio of 10:90 and was run isocratically at a flow rate of 1.44 mL min⁻¹. Samples were separated using Phenomenex® Luna C18, 5 m (250 $\times$ 4.6 mm) chromatographic column and detected using a 200 nm UV detector.

Callus and Embryoid Induction

Young leaf explants were sterilised by submerging in 0.35% sodium hypochlorite (Sigma) solution for 20 min with occasional shaking, followed by immersion in a 0.02% glucose solution (Sigma). The explants were then grown on callus induction medium (a combination of basic Murashige and Skoog medium (Merck) with 0.002-11.050 mg L⁻¹ of 2,4-D (Sigma) and incubated in the dark at 26°C-27°C. Callus would form in the fourth month onwards, and the leaves associated with the growing callus were sampled every month. The callus was isolated and transferred onto an embryo induction medium [basic medium MS with 0.002-11.050 mg L⁻¹ of 2,4-D and 0.002-11.050 mg L⁻¹ of benzyl amino purine (BAP) (Sigma)]. Embryoids would appear at different times. Observation of the callus and embryo induction was done weekly

for 12 months, and the number and emergence time of the embryos were recorded (Haska *et al.*, 2019).

Statistical Analysis

For each clone, only three leaf samples (replicates) were taken due to the limited quantity of young palm leaves. Not all sampled leaves indicated endogenous hormone production on HPLC analysis. Thus, statistical analysis was carried out on the clones identified as containing endogenous hormones. The obtained data were normalised and later analysed using the IBM SPSS program (Version 26). Results were further tested for significance using the Duncan Multiple Range Test at the 5% confidence level.

RESULTS AND DISCUSSION

Endogenous Hormone Content

This study analysed the endogenous hormones in the explants of young palm leaves to determine the effect of hormones on callus and embryoid induction. The high chromatographic peak for all the nine hormone standards (Sigma) were obtained, namely 3-methyl-trans-2-butenylamino purine (zeatin), 6-furfuryl amino purine (kinetin), gibberellic acid (GA_3), indole-3-acetic acid (IAA), BAP, abscisic acid (ABA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), and naphthalene acetic acid (NAA). The results of the HPLC measurement of the palm leaf extract samples were referred to as the standard chromatogram (Figure 2a).

HPLC analysis on clone-1 (Figure 2b) dan clone-2 (Figure 2c) showed that not all hormones were identified except zeatin, ABA, and IBA which were detected. These unidentified hormone types seemed to have very low content and, thus, were not readable. Conversely, the chromatogram of clone-3 (Figure 2d) and clone-4 (Figure 2e) showed all types of endogenous hormones analysed. These results indicated that the clone genotypes showed variations in the type and concentration of endogenous hormones, and this possibly had an effect on growth and development in the field or *in vitro* tissue culture (Correa *et al.*, 2016).

Plant parts contain different endogenous hormones, which depend on the plant organ function and the season (Vedenicheva *et al.*, 2018). The content of endogenous hormones, ABA and zeatin in *Curcuma alismatifolia* Gagnep. plant increases outside the growing season and is stored in the leaves, rhizomes and roots (Hongpakdee *et al.*, 2010). The endogenous hormone cytokinin was found to play a role in the anthesis of macadamia

crosses (Trueman, 2010). In the initiation stage of the pineapple flower, it was found that the endogenous hormone content of zeatin, IAA, and 2-isopentenyl adenine (2ip) was low, whereas that of ABA and GA_3 was high (Sheng-hui *et al.*, 2010). In this study, the endogenous hormones zeatin, ABA, and IBA were found in clone-1, clone-2, clone-3 and clone-4 young palm oil leaves. The concentration of the hormones in each sample analysed was not the same, especially the standard deviations ($\pm$ sd) in clone-1 and clone-2 which were quite high. Similar results were reported, in which only IAA and ABA were found in the leaf explants of *Pennisetum purpureum* Schum. (Rajasekaran *et al.*, 1987).

The graphs of the zeatin content show high standard deviations (Figure 3) because not all the individual samples of the young palm leaves show quantifiable hormone concentration on HPLC analysis. The highest average zeatin content was found in the young leaf explants of clone-1 (97.70 mg L^{-1}) and clone-2 (80.16 mg L^{-1}) (Figure 3). Zeatin is found in many plants and a certain period such as after pollination for fruit formation (Vedenicheva *et al.*, 2018). High zeatin content was also found in etiolated plants (Grzyb *et al.*, 2017). Besides zeatin, the highest ABA was found in clone-1 (6.72 mg L^{-1}) and clone-2 (6.83 mg L^{-1}).

The endogenous hormone ABA is found in many plants and plays a role in the induction of dormancy, inhibition of germination, stomata regulation and abiotic stress resistance (Finkelstein, 2013). Certain doses of ABA help plants grow, whereas in high doses it inhibits growth (Finkelstein, 2013). Additionally, ABA is also involved in eliminating viruses (Alazem *et al.*, 2014; Alazem and Lin, 2017). The highest concentration of the endogenous hormone IBA was found in clone-3 (10.53 mg L^{-1}) and clone-4 (5.90 mg L^{-1}). IBA is an auxin group hormone that has not been widely discussed in various endogenous hormone research.

The other endogenous hormones such as kinetin, GA_3 , IAA, BAP, 2,4-D and NAA were only found in the young leaf samples of clone-3 and clone-4 (Figure 4). Endogenous hormones not detected in this study do not mean it is not present, but it could be due to their low content, below the minimum detectable limit of the HPLC. Clone-3 and clone-4 contained various endogenous hormones. Kinetin, zeatin, and BAP are cytokinin group that plays a role in cell division and plant growth support. Endogenous cytokinin helps the embryogenesis process of young leaf explants (Grzyb *et al.*, 2017).

Generally, the content of 2,4-D was found to be higher than that of kinetin, GA_3 , IAA, BAP and NAA (Figure 4). The highest 2,4-D content was found in clone-3 (2.53 mg L^{-1}) and clone-4 (2.58 mg L^{-1}) (Figure 4); and it was not detected

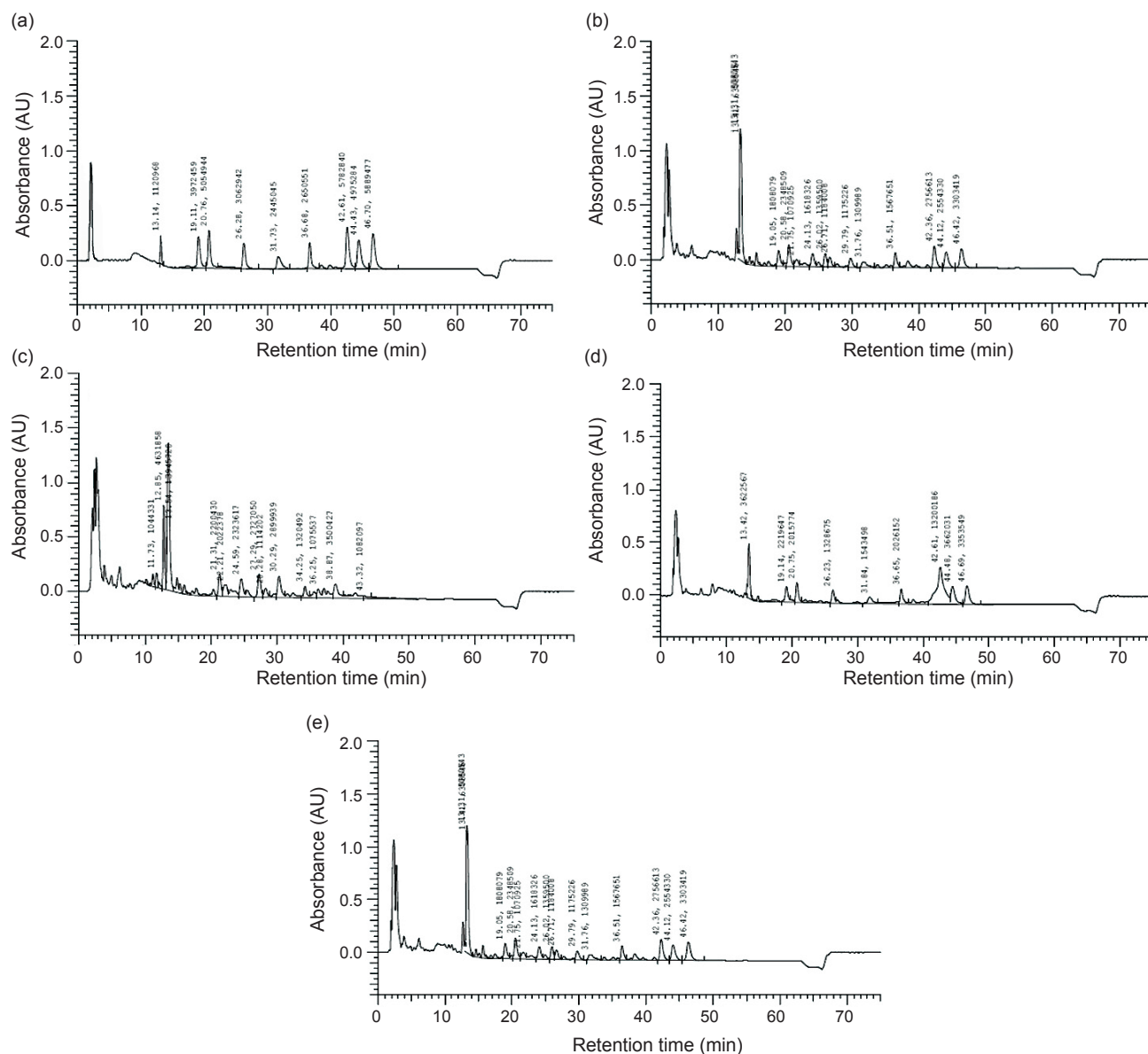


Figure 2. Chromatogram representatives of the endogenous hormone analysis on the oil palm young leaf explants: (a) plant hormone standards, (b) clone-1, (c) clone-2, (d) clone-3, and (e) clone-4.

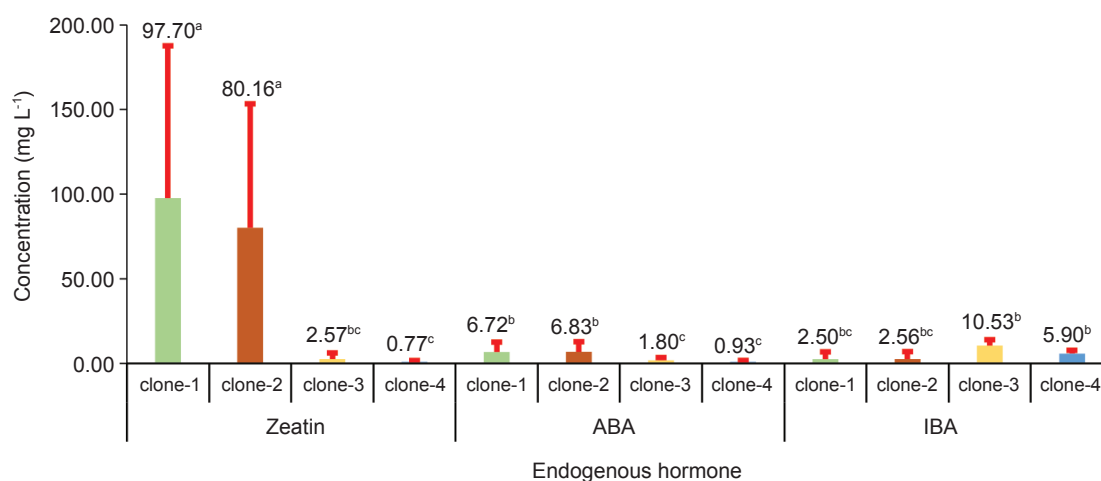


Figure 3. Endogenous hormone content of zeatin, ABA, and IBA in the oil palm young leaves of clone-1, clone-2, clone-3, and clone-4. The graph shows the mean values ($\pm$ sd) of 3 replicates, followed by the Duncan test with a 0.05 confidence level. Numerical values with different letters indicate significant differences (Zeatin: 3-methyl-trans-2-butenylamino purin, ABA: abscisic acid, and IBA: indole-3-butyric acid).

in clone-1 and clone-2. Kinetin, GA₃, IAA, BAP, and NAA were present in all the clones with concentrations that were not significantly different ($p>0.05$) (Figure 4). Even at low concentrations, endogenous hormones influence plant physiology.

The endogenous hormone IAA or auxin is synthesised in the shoots and increases in concentration in non-merismatic tissues (Ogunyale *et al.*, 2014). IBA is an endogenous hormone of the auxin group which is produced in the shoots, distributed throughout the plant body, and is effective in assisting root induction. BAP belongs to a group of cytokinin that plays a role in cell division to support plant growth. GA₃ is often found in plants and facilitates normal plant growth by accelerating cell division. GA₃ promotes cell elongation, thereby increasing the flowering process and leaf weight as well as triggering seedless fruit production (parthenocarpy).

Effect of Endogenous Hormones on Callus Induction

Explant samples of young oil palm leaves were grown on modified callus induction media, which generally consisted of macronutrients, micronutrients, vitamins, amino acids and

measurable growth regulators (Haska *et al.*, 2019; Yunita *et al.*, 2011). But in reality, the problem of non-uniform induction persists. Propagation of oil palm clones through the somatic embryogenesis method indirectly begins with the callus induction stage. Callus formation is generally assisted by the addition of auxin group hormones. Auxin hormone which is often used for callus induction is 2,4-D due to its easy absorption by plants, being hardly degradable, morphogenetic activity enhancement and facilitative role in somatic cell embryogenesis (Asra *et al.*, 2020). The hormones' ability to induce callus formation is influenced by the concentration, types of explants and plants used in the culture.

The highest percentage of callus induction was produced by clone-3 (14.89%), then clone-4 (5.65%), whereas clone-1 and clone-2 produced a lower percentage of callus (around 0.45%-0.98%) (Figure 5). The callus induction ability of all the clones was not the same, presumably due to the genotypic factor.

Every plant has endogenous hormones present throughout its organs, of which shoots and roots generally contain the highest hormone content. Therefore, the selection of explant sources in tissue culture is very crucial. Another factor that facilitates callus induction is the synthetic hormones added to maximise callus formation since both

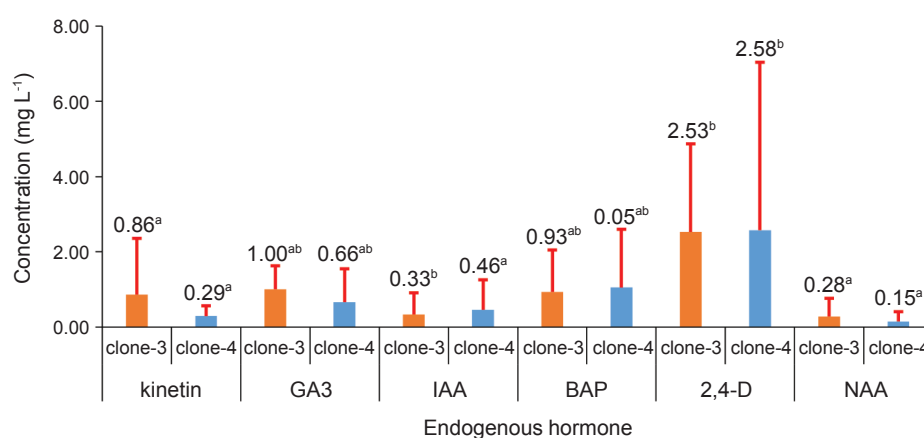


Figure 4. Endogenous hormone content of kinetin, GA₃, IAA, BAP, 2,4-D, and NAA in the oil palm young leaves of clone-1, clone-2, clone-3, and clone-4. The graph shows the mean values ($\pm$ sd) of three replicates, followed by the Duncan test with a 0.05 confidence level. Numerical values with different letters indicate significant differences. (kinetin: 6-furfuryl amino purine, GA₃: gibberellic acid, IAA: indole-3-acetic acid, BAP: benzyl amino purine, 2,4-D: 2,4-dichlorophenoxyacetic acid, and NAA: naphthalene acetic acid).

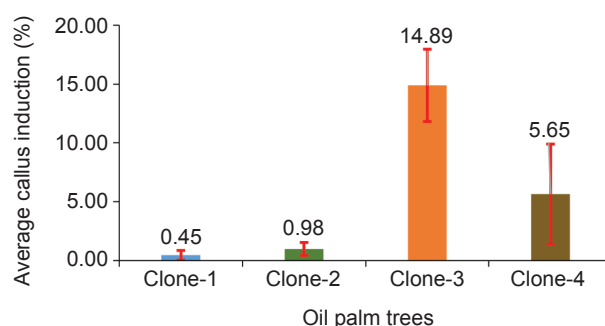


Figure 5. Percentage of callus induction of four oil palm clones.

endogenous and synthetic hormones are thought to be complementary to each other in inducing callus formation (Ayil-Gutierrez, 2013). Callus induction is generally facilitated by the hormone auxin, and 2,4-D, picloram, as well as NAA, which are the synthetic auxins commonly used in oil palm tissue culture (Reflini, 2017). Endogenous hormones zeatin and 2ip affect the formation and proliferation of embryogenic callus (Vedenicheva *et al.*, 2018). Hazelnut embryogenic callus was formed due to the balanced presence of endogenous hormones IAA, ABA, 2ip, and zeatin (Centeno *et al.*, 1997). Endogenous hormone balance between auxin and cytokinin helped the formation of *Coffea canephara* embryogenic callus (Avilez-Montalvo *et al.*, 2022). In addition to hormonal influence, callus is also formed due to a wound on the explant surface (Chen *et al.*, 2016).

The results of observations on oil palm tissue culture found that callus induction ability did not correlate with embryoid induction ability. This was presumably due to differences in callus quality produced in each clone. A quality callus is an embryogenic callus that has the potential to produce embryoids. The callus produced by clone-3 and clone-4 was more embryogenic (Figure 6). The embryogenic callus formed was thought to be related to the endogenous hormone content

of the initial explant. Embryogenic callus had characteristics such as yellowish white colour, being solid and crumbling (Figure 6). Embryogenic callus colour is influenced by endogenous hormones, where low ABA concentrations affect callus colour and formation and embryoid germination (Jimenez and Bangerth, 2000; Vedenicheva *et al.*, 2018). The high endogenous hormone content of ABA in embryogenic calluses has the potential to produce embryoids and generate new plants in greater numbers. Embryogenic callus has the potential to support the mass production of oil palm ramets (Reflini, 2017).

Non-embryogenic callus was found in the induced leaves of clone-1 and clone-2, with the characteristics of being white, soft and slightly watery (Figure 7). Non-embryogenic callus has less potential to produce embryoids. In a previous report, the high content of endogenous hormones GA₃ and ABA coupled with low IAA had the potential to generate a non-embryogenic callus of *Ormosia henryi* Prain. culture (Wu *et al.*, 2020). High ABA content could trigger embryogenic callus formation, and low ABA concentrations increased non-embryogenic callus (Vedenicheva *et al.*, 2018). In addition to its role in callus induction and embryoid formation, the endogenous hormone ABA was also demonstrated to prevent viral attacks (Grzyb *et al.*, 2017).

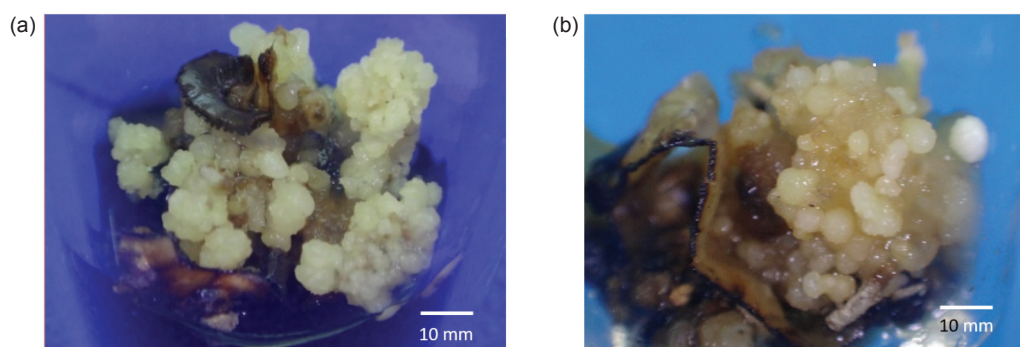


Figure 6. Leaves with embryogenic callus of (a) clone-3, and (b) clone-4.

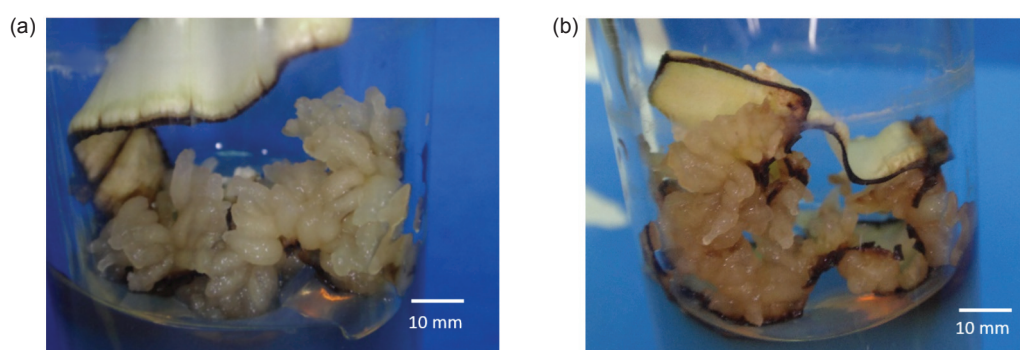


Figure 7. Leaves with non-embryogenic callus of (a) clone-1, and (b) clone-2.

TABLE 1. RESULT OF ENDOGENOUS HORMONE CORRELATION ANALYSIS ON CALLUS INDUCTION (R^2)

Endogenous hormone	Correlation value (R^2)			
	Clone-1	Clone-2	Clone-3	Clone-4
Zeatin	0.3082	0.4659	0.6218	0.3140
Kinetin			0.7500	0.7595
GA			0.2286	0.4709
IAA			0.7500	0.4395
BAP			0.6808	0.4503
ABA	0.8232	0.9371	0.0933	0.9983
IBA	0.0568	0.1702	0.0250	0.8996
2,4-D			0.3410	0.4395
NAA			0.7500	0.4395

The selection of the initial explant sample is very important because it will affect the callus induction ability and embryoid formation. Oil palm tissue culture uses samples of young leaves, which are not only merismatic but also contain endogenous hormones. In this study, the types of endogenous hormones found in clone-3 and clone-4 were more than those in clone-1 and clone-2.

The correlation of the effect of endogenous hormones on callus formation was analysed using a scatter plot in Excel, and R^2 data were obtained (Table 1). The results of the analysis were based on R^2 data; endogenous hormone ABA was correlated with callus formation in clone-1 (R^2 ; 83.32%), clone-2 (R^2 ; 93.71%) and clone-4 (R^2 ; 99.83%). Likewise, for the hormone kinetin, there was a correlation between clone-3 (R^2 ; 75.00%) and clone-4 (R^2 ; 75.95%). The correlation analysis data does not ensure that only a few endogenous hormones affect callus formation, but a combination of several other endogenous hormones had affected the formation and quality of the resulting callus.

Embryogenic calluses can be formed due to a combination of endogenous hormones and synthetic hormones added to the culture medium. The addition of synthetic hormones in culture media must be adjusted and regulated to avoid somaclonal variations. The use of synthetic hormones such as 2,4-D in high doses can cause mutations and fruit abnormalities; thus, their use must be measured in low doses (Saptari and Sumaryono, 2018). The information about the presence of endogenous 2,4-D should prevent the addition of synthetic 2,4-D at high concentrations, which can cause variations or mantled fruit formation.

Effect of Endogenous Hormones on Embryoid Induction

The resulting callus was then transferred to the embryo induction medium. In this medium, not all subcultured calluses could produce embryoids. The formation of embryoid depends on the callus cells that had formed previously. If the callus cells

formed are embryogenic, the potential for embryoid formation is very high. In this study, the callus formed had the potential to produce a low number of embryoids. The percentage of induced embryoids for each clone was not the same (Figure 8). The callus from clone-3 and clone-4 produced embryoids, whereas the callus from clone-1 and clone-2 did not produce any embryoids. The induction ability of embryoids produced by clone-4 reached 44.45% (45 tubes) and clone-3 was about 9.49% (18 tubes) (Figure 8).

Oil palm somatic embryos that have the potential to differentiate into plantlets had the following characteristics: Yellowish-white, dense embryos, loose embryos (not sticking together) and regular shaped (Figure 9).

Endogenous hormones are believed to play a role in the formation of the embryo. The results of the analysis of young oil palm leaves of clone-3 and clone-4 revealed nine types of endogenous hormones in various concentrations. Several endogenous hormones were found to correlate with embryoid formation. Endogenous hormones zeatin (R^2 ; 98.93%) and ABA (R^2 ; 77.50%) correlated in helping the formation of embryoids in clone-3 (Figure 10), whereas the hormones IBA (R^2 ; 85.18%) and ABA (R^2 ; 98.70%) correlated in the formation of embryoid in clone-4 (Figure 10). The combination of various types of endogenous hormones collaborated in the formation of potential embryoids. The high content of ABA helped in embryo differentiation, while the addition of low IAA and zeatin maintained the embryogenic potential in Korean pine callus (Liang *et al.*, 2022). The content of endogenous hormones ABA, IAA, and GA₃ in *Ormosia henryi* Prain. decrease with the process of embryogenesis (Wu *et al.*, 2020).

The auxin and cytokinin found in clone-3 and clone-4 in combination with synthetic hormones in culture media were able to support the formation of embryogenic and embryoid calluses. The addition of synthetic hormones into the media increases the concentration of endogenous hormones in cells, hence triggering tissue growth and development (Gaba, 2005). Cytokinin hormone is needed in the

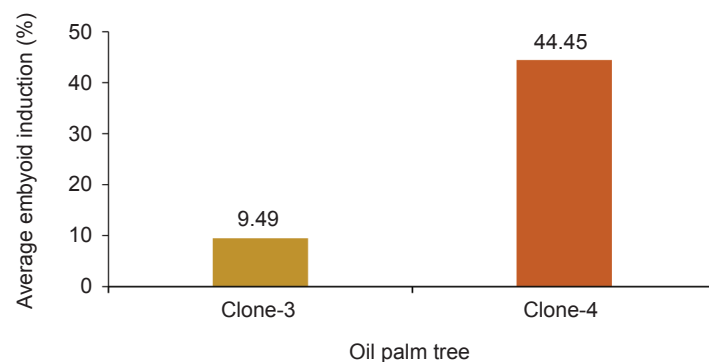


Figure 8. Percentage of somatic embryo formation from (a) clone-3, and (b) clone-4.

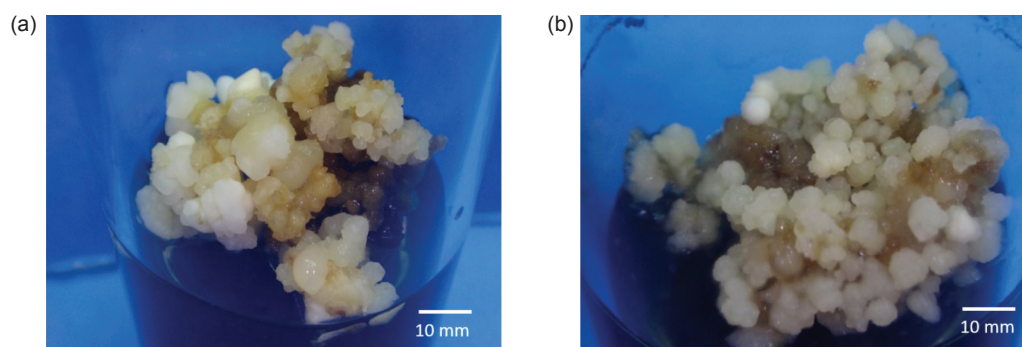


Figure 9. Embryogenic callus of prospective embryoids of (a) clone-3, and (b) clone-4.

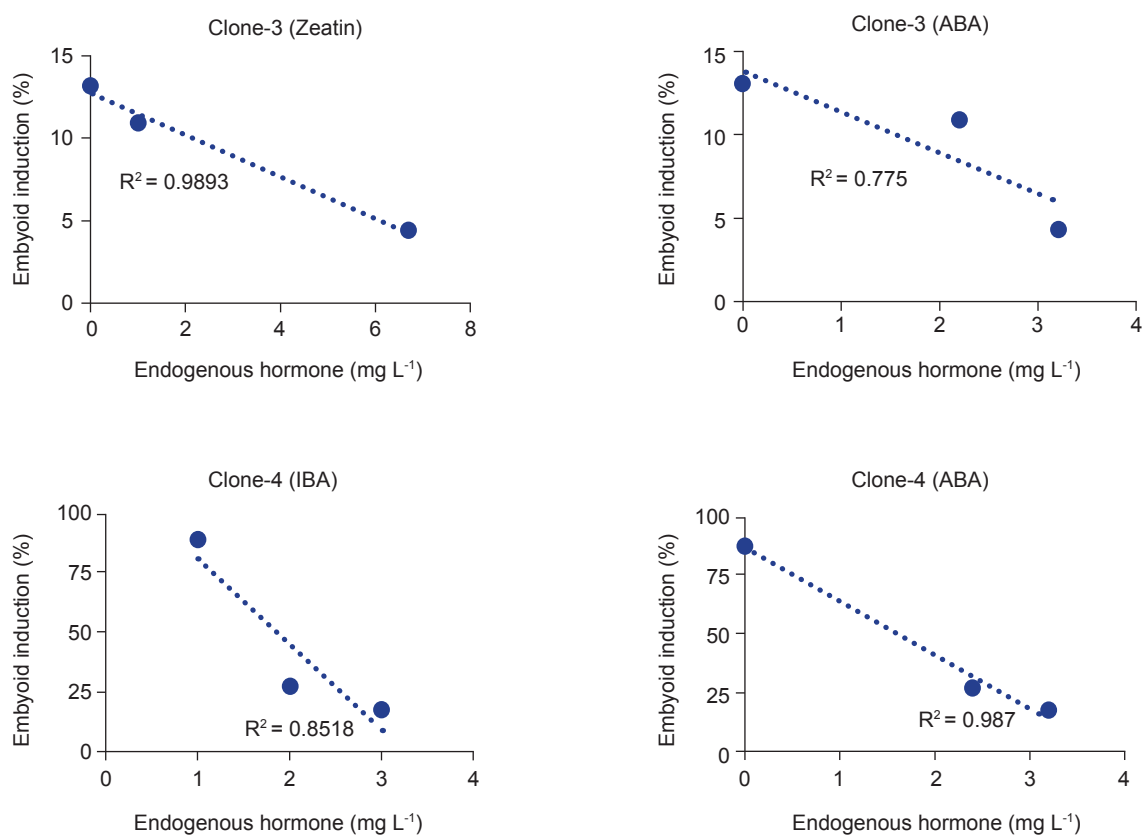


Figure 10. Correlation of endogenous hormones with the percentage of embryo induction in clone-3, and clone-4.

formation of embryoids. The types of cytokinin added to oil palm tissue culture media were BAP, 2iP and kinetin (Weckx *et al.*, 2019). Cytokinin hormones also promote the regeneration of embryos into shoots and plantlets (Aslam *et al.*, 2011). The addition of BAP-type cytokinins at a concentration of 1 M was able to increase the germination and development of somatic embryos (de Olivera *et al.*, 2016).

The addition of synthetic hormones into the culture medium must consider the presence of endogenous hormones in the selected explant source. Excessive accumulation of hormones in plant tissues and culture media can cause abnormalities problems (Weckx *et al.*, 2019). Growth regulators or hormones do not have a direct effect as mutagens; however, if added excessively, they can stimulate fast irregular growth, causing abnormalities (Bairu *et al.*, 2011). Other factors that can trigger abnormalities are too high concentrations of 2,4-D and repeated subculture processes (Ernayunita *et al.*, 2017). In addition to functioning as a growth regulator, 2,4-D turned out to be toxic to plants when used in high concentrations (Vasil and Vasil, 1972). The use of high 2,4-D can cause physiological and biochemical disturbances in plants resulting in abnormalities. Thus, the supplementation of synthetic hormones must be carefully measured because it can trigger abnormalities when combined with high content of endogenous hormones. Initial information about the content of endogenous hormones is important; this is because the process of *in vitro* propagation of oil palm plants takes a long time. The sampling of young leaf explants to produce plantlets takes about 3-4 years. This research is expected to provide information in determining the type of hormone and the concentration of synthetic hormone needed, especially in the oil palm clonal propagation activities.

CONCLUSION

The type and content of endogenous hormones in the young leaves of each oil palm clone were found to be different. Endogenous hormones in young oil palm leaves affected the callus and embryoid induction ability. The analysis of young leaves from clone-1 and clone-2 showed three types of endogenous hormones (zeatin, ABA and IBA), whereas clone-3 and clone-4 contained nine types of endogenous hormones (zeatin, kinetin, GA₃, IAA, BAP, ABA, IBA, 2,4-D and NAA). Young explants of oil palm leaves containing high zeatin and ABA triggered the formation of non-embryogenic callus, while the young leaves containing 2,4-D and BAP demonstrated the potential to produce embryogenic callus. The presence of endogenous hormone 2,4-D in leaf explants and the addition of synthetic hormone

2,4-D around 0.002-11.050 mg L⁻¹ in culture media induced the formation of embryogenic callus and had the potential to form embryoids and produce plantlets. The right and measurable combination of endogenous and synthetic hormones can support the clonal production of oil palm.

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AN EXPERIMENTAL STUDY ON THE EFFICIENCY OF A PALM MESOCARP SEPARATION MACHINE

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ABSTRACT

A bench-top machine would be suitable for farmers to separate palm mesocarp from palm kernels on a small scale. The purpose of this study was to conduct an experimental investigation on the efficiency of such a machine for palm mesocarp separation from the palm kernels with the following operational steps: (1) developing a separation machine having a pressing force measurement by upgrading an existing palm mesocarp separation machine; (2) conducting an experiment to determine a suitable operating point; and (3) examining the efficiency of the newly developed separation machine and analysing the extracted palm oil properties. The results show that suitable weight loadings for the cases without heating, with steaming, and with roasting are 1000, 1500 and 1500 g, respectively, whereas the optimal pressing distance is 19 cm. Deterioration Bleachability Index (DOBI) of palm oil extracted from oil palm mesocarp in the cases of no heating, steaming and roasting are 2.99, 3.68 and 4.15, respectively. In addition, the free fatty acid (FFA) in the cases of no heating, steaming and roasting are 3.89%, 3.14% and 2.72%, respectively. Therefore, DOBI and FFA levels satisfy the standard requirements for DOBI is not less than 2.00 and for FFA is not more than 5.00%.

Keywords: oil palm, palm mesocarp, separation machine.

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INTRODUCTION

Oil palm is an important economic plant that plays a significant role to the availability of vegetable oil for human consumption (Basiron, 2007; Lai *et al.*, 2012). It is also the main raw material for producing biodiesel in Southeast Asia (Mukherjee and Sovacool, 2014; Purnomo *et al.*, 2020). This economic plant provides income to farmers, large and small farms, and entrepreneurs in the palm oil industry, including the palm oil mills that extract the oil from the fruit (Corley and Tinker, 2008; Lai *et al.*, 2012; Poku, 2002). The Thai government has continuously provided encouragement and support to this industry in many aspects, for instance by encouraging farmers to extend their plantation areas and to plant better oil palm varieties with increased yields. The

government has also supported the entire cycle of research and development on various aspects related to palm oil production (Chaisongkram, 2019).

In addition, palm oil production technology has been developed further and upgraded, along with the encouragement to build low-cost small-scale palm oil mills in various communities, for small-scale production without the need for a large investment (Chaisongkram, 2019; Ekine and Onu, 2009; Ilechie *et al.*, 2011; Poku, 2002). A high-technology palm oil pressing machine can be used to separate palm mesocarp from the palm kernels prior to oil pressing. Many studies have designed and implemented various devices to increase the efficiency of producing palm oil, for use in small-scale palm oil mills, or in community palm oil mills. For instance, Sopajarn and Thongwichean (2017) designed and built a centrifuge to separate palm mesocarp from palm kernels, especially from dropped loose fruit which is a palm fruit that separates from a palm bunch, without the need for steaming prior to this separation.

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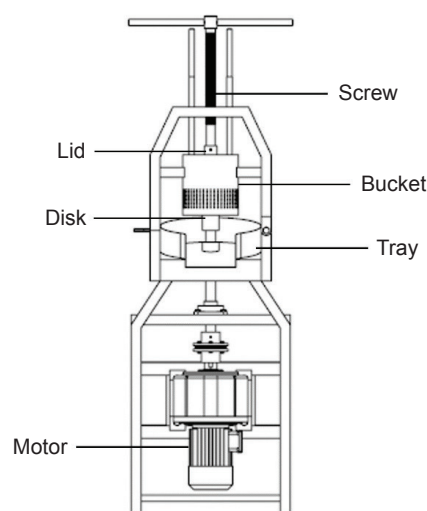
The centrifuge is able to efficiently separate fresh palm mesocarp from palm kernels, with a small amount of the palm kernels broken and some mesocarp leftovers after the separation. Similarly, Pukapak *et al.* (2015) also designed and built a stripping machine to separate palm kernels from oil palm fruit, functioning efficiently at 483 rounds/min operating speed, and with a peak separation purity of 91.76%. Khalid and Shuib (2017) fabricated and tested the performance of oil palm loose fruits separating machines. It was found that the machine can separate the loose fruits from the trash effectively with 98.90 % cleanliness. Alam *et al.* (2020) developed a manually operated palm oil extraction machine to press the pulp of palm fruit to extract oil. Tamrin *et al.* (2020) designed and made prototypes of palm seed sorting machines. Okoko *et al.* (2017) designed, fabricated and tested a fragmentation unit to enhance palm kernel separation by a dry method, using a designed fragmentation unit. Onyekachi and Nwankwojike (2020) analysed and compared the potentials of recently developed indigenous dry process based palm kernel shell separation systems to select the best one for adoption by small scale processors in Nigeria. Simultaneously, Mueangdee (2012) designed and built a separation machine to divide palm mesocarp from palm kernels, and this machine has been optimised for small-scale farmers and community palm oil industry. It is able to entirely separate the mesocarp from palm kernels. However, the earlier versions of this machine are unable to measure the pressing force in the machine while it is operating.

Therefore, this study developed a palm mesocarp separation machine with force sensors by upgrading the existing device for separating palm mesocarp from palm kernels. The developed machine is able to measure the pressing force while it is functioning, so that a suitable force for separating the mesocarp from the palm kernels can be determined. The suitable force is the maximum force that can be applied on the upper disk to separate palm mesocarp from palm kernels based on the criteria of no broken palm kernels before finishing the process of separation.

MATERIALS AND METHODS

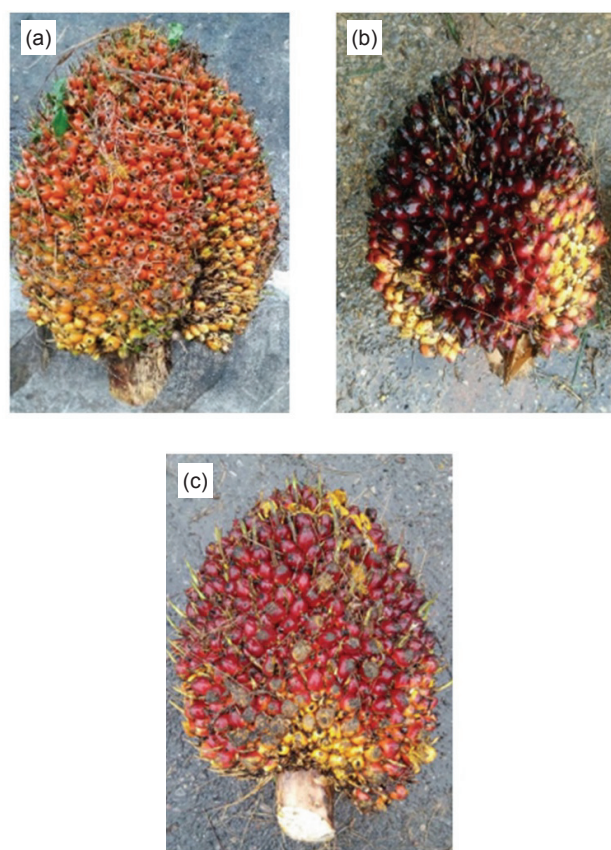
Materials

The materials used in this research were as follows: (1) A machine to separate mesocarp from palm kernels (Mueangdee, 2012; Mueangdee and Prasertsan, 2014; 2015), as shown in Figure 1; (2) Oil palm fruits of varieties Suratthani 1, Suratthani 2 and Suratthani 3, as shown in Figure 2 in different shapes and colours. In addition to being physically different, Suratthani 2 provides the highest annual fresh fruit branch yield of 22.6 t/ha when compared with Suratthani 121.5 t/ha



Source: Mueangdee (2012); Mueangdee and Prasertsan (2014; 2015).

Figure 1. Drawing of a bench-top machine for oil palm mesocarp separation.



Source: Ministry of Agriculture and Cooperatives (2011; 2020).

Figure 2. Palm fruit bunches of three varieties: (a) Suratthani 1, (b) Suratthani 2, and (c) Suratthani 3.

and Suratthani 318.4 t/ha. Regarding the percentage of oil extraction rate Suratthani 1, 2 and 3 can provide 26%, 23% and 27%, respectively (Ministry of Agriculture and Cooperatives (2020); (3) Devices

for measuring distance, speed, force, weight, and time. The distance is measured by a metal ruler. The speed is measured by a digital tachometer. The force is measured by a load cell. The time is measured by a stopwatch; and (4) Devices for heating the palm fruit are an oven and a steamer.

Methods

There were four main stages in the study: (1) Developing a separation machine with runtime pressing force measurement. A machine for separating palm mesocarp from palm kernels, with pressing force measurement, was established by installing to the device a tool for measuring the distance between the upper disk surface and the lower one, as well as a sensor to record the force; (2) Setting up an optimised value. In order to look for a suitable operating point for separating mesocarp from palm kernels in the separation machine, equipped with a pressing force measurement, the following three steps were conducted (Figure 3).

- (i) Sampling of loose fruits from fruit bunches of three palm varieties, Suratthani 1, Suratthani 2 and Suratthani 3, for use in the experiments.
- (ii) Finding an appropriate weight for operation, by use of the implemented pressing force measurement.
- (iii) Finding an appropriate distance between the upper and lower disks;

(3) Test of the machine efficiency. The separation machine equipped with a pressing force measurement was tested at the operating point obtained in stage 2. The next step was to record the data on force, speed, distance between disks, duration, palm mesocarp weight, palm mesocarp percentage, weight of palm kernels, and palm kernels percentage. Further, analytical results were obtained on properties of the palm oil extracted

from separated mesocarp for the palm varieties Suratthani 1, Suratthani 2 and Suratthani 3, along with three preconditioning methods before separation: (a) no heating; (b) steaming for 45 min; and (c) roasting at temperature of 100 for 120 min; and (4) Statistical analysis. Descriptive statistics (percentage, mean, and standard deviation) were assessed along with comparison of means by one-way analysis of variance. The indicated differences of means were confirmed by using Tukey's HSD test at significance requirement $p < 0.05$.

RESULTS AND DISCUSSION

Developing the Separation Machine Equipped with a Pressing Measurement

Figure 4 shows a schematic of the machine with a pressing force measurement installed. The set-up has a tool for measuring the length in centimetres to observe the pressing distance between the upper and lower disk surfaces, and a load cell to measure the force.

Experiments for Determining Optimal Operating Point to Separate Mesocarp from Palm Kernels

The results to determine a suitable operating point comprised the following data: (1) The results for the three palm varieties with three alternative preconditioned states: Not heated, steamed, and roasted; (2) The results on proper weight loading for the palm fruit; and (3) The results on the distance between upper and lower disk surfaces.

Experimental outcomes on separating mesocarp from palm kernels for the three palm varieties and the steamed preconditioning method. 1 kg of palm fruit for each variety was subjected to the separation and the results are summarised in Table 1.

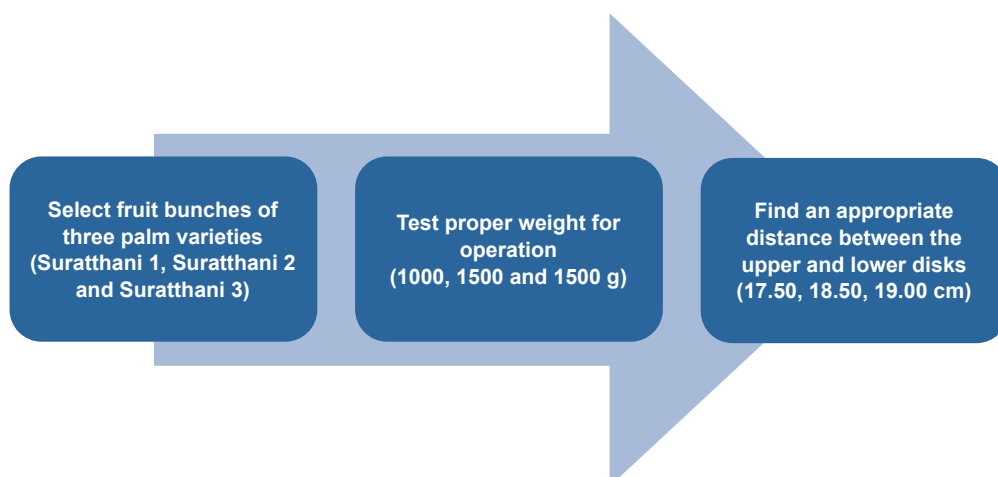


Figure 3. Experimental setup and procedure.

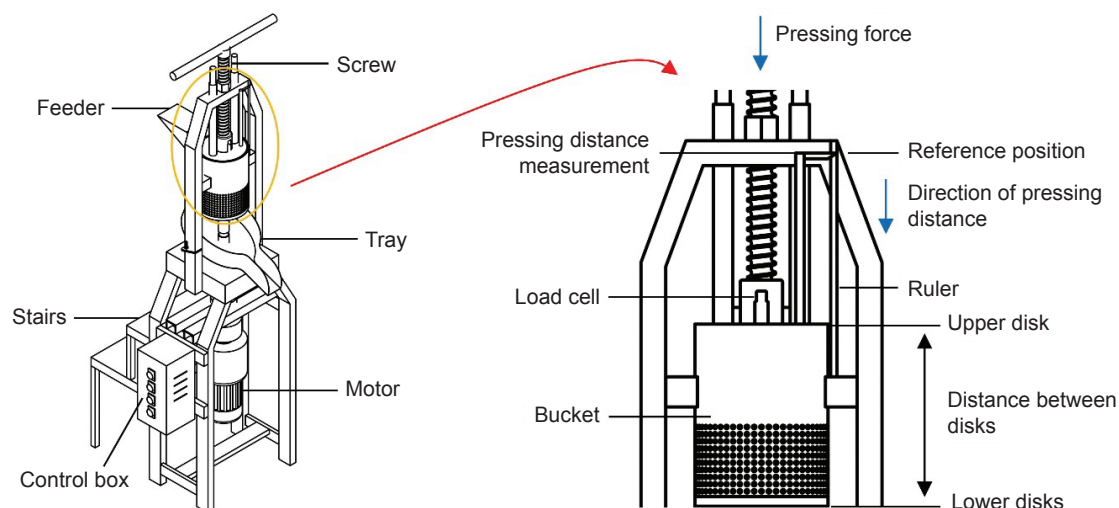


Figure 4. Drawing of the machine equipped with pressing measurements.

TABLE 1. EXPERIMENTAL RESULTS FROM SEPARATING MESOCARP FROM PALM KERNELS FOR THREE VARIETIES, SURATTHANI 1, SURATTHANI 2 AND SURATTHANI 3

Experiment	Suratthani 1		Suratthani 2		Suratthani 3	
Force (N)	1 882.88 ^b	35.36	1 961.33 ^b	19.61	1 719.43 ^a	49.36
Speed (RPM)	9.89 ^b	0.05	9.90 ^b	0.03	9.80 ^a	0.01
Distance between disks (cm)	2.23 ^a	0.11	2.10 ^a	0.10	2.23 ^a	0.06
Duration (min)	1.35 ^a	0.01	1.32 ^a	0.01	1.33 ^a	0.02
Palm mesocarp weight (kg)	0.63 ^{ab}	0.01	0.64 ^b	0.02	0.60 ^a	0.01
Palm mesocarp percentage	69.90 ^a	1.79	72.04 ^a	2.94	68.07 ^a	2.10
Palm kernels weight (kg)	0.27 ^a	0.02	0.25 ^a	0.03	0.28 ^a	0.02
Palm kernel percentage	30.10 ^a	1.79	27.96 ^a	2.94	31.93 ^a	2.10

Note: The values are given as mean $\pm$ standard deviation; different superscripts in the same row indicate significant differences ($p < 0.05$) upon testing by using Tukey's HSD.

The palm fruits have no significant difference in mean force for Suratthani 1 and 2 varieties, yet Suratthani 3 has a significant difference in mean force. Moreover, the mean of revolving speed for Suratthani 3 differs from the others. The average percentages of palm mesocarp did not differ between the varieties. The analysis of palm mesocarp weight, separated from palm kernels, showed no difference between Suratthani 1 and 2 varieties or Suratthani 1 and 3 varieties. So, only Suratthani 1 is used in further experiments, as shown in Table 1. The final recovery of mesocarp and kernel based on starting mass of 1 kg has presented in Table 1 and Figure 5. It can be clearly seen that mesocarp separation is a complete separation for the steaming precondition as shown in Figure 5.

Experimental outcomes for optimising the weight. From the experiments to look for the appropriate weight to use in separating palm mesocarp of Suratthani 1 variety from the palm kernels,

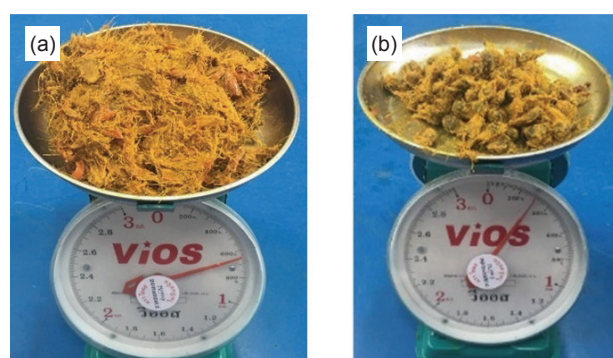


Figure 5. The (a) separated mesocarp and (b) palm kernels for the steaming precondition.

without heating, or with steaming or roasting, the results reveal the proper weight loadings as 1000, 1500 and 1500 g, respectively, as shown in Table 2.

The results on proper pressing distance. The results on separating Suratthani 1 palm mesocarp from

palm kernels without heating, or with steaming or roasting, show that the proper pressing distance is 19.00 cm. The details are given in *Table 3*.

The Experimental Results on Efficiency

The outcomes on the separation of Suratthani 1 mesocarp from palm kernels without heating, or with steaming or roasting, using weight loadings of 1000, 1500 and 1500 g and the pressing distance of 19.00 cm reveal, as shown in *Table 4*, that the mean palm mesocarp fractions were at 56.59%, 70.31% and 55.72% while the palm kernels contributed 43.41%, 29.69% and 44.29%, respectively (*Figure 6*). These figures are similar to the mean of mesocarp and the one of palm kernels obtained from the mesocarp separation from palm kernels without heating, or with steaming or roasting.

Upon subjecting the obtained mesocarp to palm oil extraction and conducting a palm oil quality analysis, it was found that the DOBI of palm oil without heating, or with steaming or roasting was 2.99, 3.68 and 4.15 ppm, while the FFA values

were 3.89%, 3.14% and 2.72%, respectively. These are consistent with the set standards. The palm oil quality analysis was conducted under the standard of the test methods at Surat Thani Palm Oil Research Center, Field Crops Research Institute, Department of Agriculture, Thailand. The results are summarised in *Table 5*.

CONCLUSION

The study improved and demonstrated an existing mesocarp separation machine by equipping it with measurement of pressing force, and of the distance between upper and lower disk surfaces. The improved machine was tested to find an appropriate operating point for separating mesocarp from palm kernels, with maximum efficiency of this separation. The suitable weight loadings for oil palm kernels without heating, steamed, or roasted, were 1000, 1500 and 1500 g, respectively, whereas the proper pressing distance was 19.00 cm. The optimised pressing distance

TABLE 2. THE EXPERIMENTAL RESULTS ON LOOKING FOR PROPER WEIGHT IN THE SEPARATION OF MESOCARP FROM PALM KERNELS FOR SURATTHANI 1

Experiment	No heating			Steaming			Roasting		
	1 000 g	1 300 g	1 500 g	1 000 g	1 300 g	1 500 g	1 000 g	1 300 g	1 500 g
Force (N)	4 560.00	5 099.46	7 433.44	1 892.68	3 314.65	3 550.01	2 402.63	3 706.91	4 530.67
Distance between disks (cm)	3.20	4.50	4.80	2.10	3.50	3.90	3.30	3.70	4.20
Duration (min)	3.02	3.42	4.31	1.36	1.54	2.19	2.05	2.52	3.40
Palm mesocarp weight (g)	570.00	654.90	747.50	640.00	711.25	961.95	490.00	622.55	822.10
Palm mesocarp (%)	57.58	54.65	54.91	71.91	63.47	74.60	54.44	51.52	60.28
Palm kernels weight (g)	420.00	543.45	613.85	250.00	409.35	327.45	410.00	585.80	541.65
Palm kernel (%)	42.42	45.35	45.09	28.09	36.53	25.40	45.56	48.48	39.72

TABLE 3. THE RESULTS ON EFFECTS OF PRESSING DISTANCE ON THE SEPARATION OF SURATTHANI 1 PALM MESOCARP FROM PALM KERNELS FOR SURATTHANI 1

Pre-treatment	Pressing distance (cm)	Palm weight (g)	1 st experiment				2 nd experiment			
			Force (N)	Duration (min)	Palm mesocarp weight (g)	Palm mesocarp percentage	Force (N)	Duration (min)	Palm mesocarp weight (g)	Palm mesocarp percentage
No heating	17.50	1 000	1 125.35	0.13	-	-	1 647.52	0.15	-	-
	18.50	1 000	2 922.38	2.10	-	-	2 671.27	2.25	-	-
	19.00	1 000	4 450.09	2.56	520.45	56.22	4 213.75	3.13	507.35	56.95
Steaming	17.50	1 500	1 157.18	0.27	-	-	1 206.22	0.25	-	-
	18.50	1 500	2 745.86	0.58	-	-	2 687.02	0.45	-	-
	19.00	1 500	3 138.13	1.49	864.35	70.97	3 089.09	2.05	910.45	69.65
Roasting	17.50	1 500	1 353.32	0.36	-	-	1 255.25	0.42	-	-
	18.50	1 500	3 012.71	1.54	-	-	3 138.13	2.15	-	-
	19.00	1 500	3 418.79	2.5	788.85	60.94	3 464.78	3.12	644.50	50.49

TABLE 4. EXPERIMENTAL RESULTS OF SEPARATING MESOCARP FROM PALM KERNELS BY USING THE DEVELOPED SEPARATION MACHINE FOR SURATTHANI 1

Method	Pressing distance (cm)	Palm weight (g)	Force (N)	Duration (min)	Palm mesocarp		Palm kernels	
					Weight (g)	%	Weight (g)	%
No heating								
1 st attempt	19.00	1 000	4 450.09	2.56	520.45	56.22	405.35	43.78
2 nd attempt	19.00	1 000	4 213.75	3.13	507.35	56.95	383.55	43.05
Mean	19.00	1 000	4 331.92	2.85	513.90	56.59	394.45	43.41
Steaming								
1 st attempt	19.00	1 500	3 138.13	1.49	864.35	70.97	353.50	29.03
2 nd attempt	19.00	1 500	3 089.09	2.05	910.45	69.65	390.65	30.35
Mean	19.00	1 500	3 113.61	1.77	887.4	70.31	372.08	29.69
Roasting								
1 st attempt	19.00	1 500	3 418.79	2.50	788.85	60.94	505.55	39.06
2 nd attempt	19.00	1 500	3 464.78	3.12	644.50	50.49	632.05	49.51
Mean	19.00	1 500	3 441.79	2.81	716.68	55.72	568.80	44.29

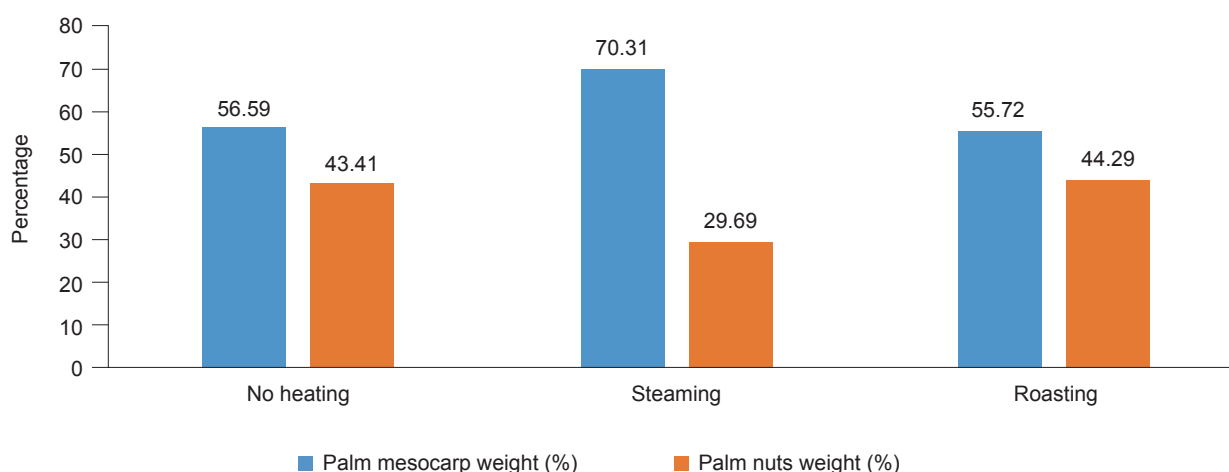


Figure 6. The mean fractions of palm mesocarp and palm kernels.

TABLE 5. RESULTS FORM THE QUALITY ANALYSIS OF PALM OIL EXTRACTED FROM MESOCARP, SEPARATED FROM PALM KERNELS BY THE DEVELOPED MACHINE FOR SURATTHANI 1

Pre-treatment	DOBI (ppm)	Vitamin A (ppm)	FFA (%)	Moisture (%)
No heating	2.99	895.04	3.89	3.23
Steaming	3.68	765.49	3.14	4.62
Roasting	4.15	779.66	2.72	1.40

Note: The set standard for DOBI is not less than 2.00 and for FFA it is not more than 5% (Johansson and Pehlgergaard, 1976; Lai *et al.*, 2012).

produced the maximum amount of palm mesocarp without breaking the palm kernels. This distance is a key factor in increasing the production while reducing losses in the separation process. In addition, quality analysis of the extracted palm oil demonstrated values of DOBI, vitamin A, FFA

and moisture being consistent with set standards: DOBI was not less than 2.00 ppm; vitamin A ranged within 500-1600 ppm; FFA was less than 5% and moisture was less than 5%. Regarding future work for this research, attention could be drawn to the design for different shapes of upper disk surface for faster separation process with higher efficiency. In addition, the experiment of the separation machine with the palm farmers should be further explored in the future study.

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ARTHROPOD FAUNA OF OIL PALM INFLORESCENCE (*Elaeis guineensis*), WITH NOTES ON THEIR ECOLOGICAL ROLES IN A PLANTATION IN TERENGGANU, MALAYSIA

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ABSTRACT

*Arthropod communities at oil palm inflorescence, specifically the insects, are known to forage for floral resources and hence act as pollinators to the palm tree. However, their activities at the inflorescence other than as pollinators are rarely explained. This study was conducted at an oil palm plantation located at Hulu Terengganu District, in Terengganu state, Peninsular Malaysia. Arthropod fauna was extracted from 15 male and female inflorescences for identification, and bottle traps were set up to capture the arthropod fauna for pollen load observations. A total of 14 insect taxa, two spiders and a millipede were recorded in this study. A high abundance of *Elaeidobius kamerunicus* was recorded and with high conspecific pollen loads on their bodies further confirming their importance as pollinators for the palm tree. Predation activity by an earwig on larvae of the insect pollinators which used the male inflorescence as a breeding site was observed in this study. Other than utilising the oil palm inflorescence as a preying and breeding ground, earwigs, ants, wasps and flies were also likely pollinators for the oil palm tree. Hence this study adds more understanding of the ecological role of arthropods in the oil palm plantations in Malaysia.*

Keywords: conspecific pollen, earwig, larvae, pollinator, predator.

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INTRODUCTION

The African oil palm, *Elaeis guineensis* originated from the African continent and is widely cultivated in Southeast Asia, particularly in Malaysia and Indonesia, the two primary producers of palm oil products in the world (Corley and Tinker, 2016). During its early introduction in Malaysia, this palm

was originally believed to be mainly pollinated by wind (Hardon and Turner, 1967; Turner, 1978), before further observations indicated that insect pollinators particularly the oil palm weevil, *Elaeidobius kamerunicus* are crucial in the pollination of this palm (Syed, 1979). Before the introduction of this weevil in Malaysia in 1980 (Basri *et al.*, 1983), assisted pollination was practised to improve the fruit yield of this important cash crop (Teo, 2015). However, assisted pollination also resulted in poor fruit sets due to the difficulty in applying pollen grains to the inner part of the oil palm inflorescence (Harun and Noor, 2002). Insects such as the hymenopteran bees and dipteran flies are the major pollinator groups for agricultural crops (Wojcik, 2021). In the case of commercial oil palm (Genus *Elaeis*), the insect pollinators are predominantly the coleopteran weevils from the Genus *Elaeidobius* (Appiah and Agyei-Dwarko, 2013; Li *et al.*, 2019; Melendez and Ponce, 2016).

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The activities of insects in oil palm inflorescences were always described in relation to their activity to forage for food (Rizali *et al.*, 2019; Sambathkumar and Ranjith, 2011; Syed, 1979). Other than foraging for the flower resources, the insect also visits the oil palm inflorescence to prey on other insects and also for appropriate living space and breeding sites (Li *et al.*, 2019; Muhammad Luqman *et al.*, 2017; Rizali *et al.*, 2019; Yue *et al.*, 2015). Other than the extensive studies on the relationship between *E. kamerunicus* and the oil palm inflorescence which has indicated its crucial role in fruit yield of the economically important palm tree (Yousefi *et al.*, 2020), further investigation on the ecological significance of the arthropod communities at the oil palm inflorescence is rarely explored (Li *et al.*, 2019). Competition between the visitors, or predation on the main pollinators, for example, could influence the fruit yield. Moreover, the pollination success of the *E. kamerunicus* population was found to be highly influenced by climatic conditions (Yousefi *et al.*, 2020).

The oil palm tree produces the male and female inflorescences alternately (Adam *et al.*, 2011; Sambathkumar and Ranjith, 2015), hence requiring pollinating agents to move the pollen grains from the male to the female inflorescence. Thus, any flower visitors could be considered pollinators, even if they are visiting for ovipositing or preying on the inflorescences. In Malaysia, other than *E. kamerunicus*, *Pyroderces* sp. (cosmeth moth) and *Thrips hawaiiensis* (Hawaiian flower thrips) are the only known native insect pollinators for the oil palm although these insects are less efficient (Wahid and Kamarudin, 1997). We believe that other arthropod flower visitors could also act as pollinators for the oil palm, hence this study was conducted to explore the potential significance of other arthropods as pollinators of the palm trees. The information gained from this study could improve our understanding of the ecosystem services provided by the arthropod fauna in the oil palm plantation, particularly in relation to the pollination success of the palm trees in Peninsular Malaysia. Hence, the knowledge of the ecological and economically important arthropod communities is valuable not only to the scientific community but also to industrial players as it enables biodiversity-friendly management in the oil palm plantation.

MATERIALS AND METHODS

Study Site

Samplings of arthropods were carried out at Ladang TDM Jerangau (hereafter Ladang Jerangau), owned by Terengganu Development

Management (TDM) Sdn. Bhd., Terengganu state in Peninsular Malaysia (Figure 1). The study site is located in Hulu Terengganu District (4°57'45"N 103°9'59"E), situated about 65 km from Kuala Terengganu. The palm trees (*E. guineensis*) selected for data collection were between 6 to 8 years old. Samplings were conducted from June 2019 until February 2020, while the work in the laboratory started in December 2019 and was completed in March 2020.

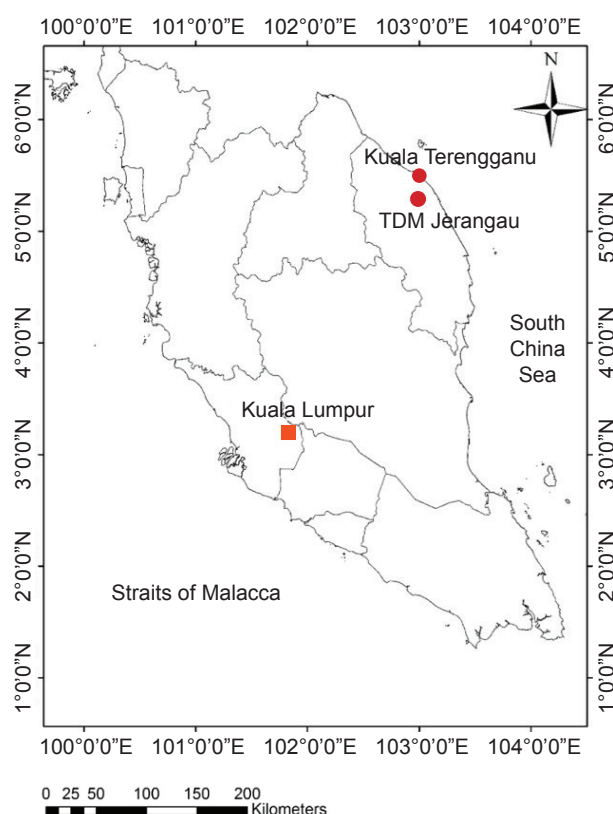


Figure 1. The location of the sampling site, TDM Jerangau (Ladang Jerangau) located in Terengganu state, Peninsular Malaysia.

Extraction of Arthropods from the Inflorescence

A total of 15 male and 15 female inflorescences were selected for the arthropod collections. These 15 inflorescences consisted of five pre-anthesis, five anthesis and five post-anthesis inflorescences. Identification of the anthesis stage was conducted based on the morphological descriptions of the inflorescence by Forero *et al.* (2012). The selected inflorescences were bagged in individual plastic bags and extracted from the trees. The inflorescences were then taken to the field station for observation. For each inflorescence, only 50% of the spikelets (male inflorescence) and rachillae (female inflorescence) were separated from the stalk and kept in plastic containers before being dissected individually under a dissecting microscope (Leica DME, Leica Microsystem [SEA]

Pte. Ltd., Malaysia). All arthropods observed from the spikelets and rachillae were then transferred into individual vials filled with a 70% ethanol solution and brought back to Universiti Malaysia Terengganu (UMT) for further identification in the laboratory. The arthropods were observed under a light microscope (CH20, Metric Optics Sdn. Bhd., Malaysia) in the General Biology Laboratory, UMT, and identification was conducted to the lowest taxonomic category possible, by referring to Gullan and Cranston (2014), Halim *et al.* (2017) and Norman *et al.* (2017).

Rearing of Larvae

Other than the adult individuals, larvae were also collected from the male inflorescence. As species identification is difficult at the larval stage, the larvae and spikelet were kept in the plastic container under ambient conditions in the General Biology Laboratory, UMT, until the larvae emerged into adults (approximately one week). The larvae were monitored daily and the adults that emerged were taken from the containers and observed under a dissecting microscope (Leica DME, Leica Microsystem [SEA] Pte. Ltd., Malaysia) for species identification.

Trappings of the Floral Visitors and Observations of Pollen Load

A modified bottle trap (Figure 2) was set up to capture the visitors of five male and female inflorescences (a trap each). Trappings were conducted for three days (anthesis day 3-5) for the male and two days (anthesis day 2-3) for the female inflorescence, during their peak anthesis time. The traps were installed at 1000 hr and checked regularly until 1100 hr for the trapped visitors, where we usually observed a high abundance of visitors at the oil palm inflorescence during this period. The trapped visitors were extracted from traps using forceps and transferred into a 1.5 mL centrifuge tube filled with 1.0 mL of 70% ethanol solution. The tubes were then taken to the laboratory for further analysis. In the laboratory, the tubes were shaken to separate the pollen grains from the visitors' bodies. For each tube, 0.5 μ L of the solution was pipetted using a micropipette, then placed on a hemocytometer slide and covered with a cover slip. Pollen grains were observed under a light microscope attached to an eyepiece camera (Dino-eye AM 423X, Anno Electronics Corporation, Taiwan) with 70x magnification up to a maximum observation at 2800x. The pollen grains were grouped as either conspecific (*i.e.*, pollen grains of *E. guineensis*) or heterospecific (*i.e.*, pollen grains other than *E. guineensis*). For each vial, the procedure was

repeated 10 times, thus the number of pollen grains was counted from 5 μ L of the ethanol solution.



Figure 2. A bottle trap was set up to catch the flower visitors of the male inflorescence. The bottom part of the plastic bottle was removed to attach it to the inflorescence. The bottles used were approximately 300 mm long and 90 mm in diameter. Trapped individuals were extracted carefully from the open tip (25 mm in diameter) using long forceps.

Statistical Analysis

The diversity of arthropods collected from the inflorescence was calculated using the Shannon formula (H'), generated from PAST (Paleontological Statistics Software) ver. 3.26 statistical software. The frequency was used to compare the distribution of arthropods and tested with Pearson's Chi-square test (Yates correction was applied when small sample size was involved, *i.e.*, $df = 1$). For the non-normally distributed data of pollen loads, the paired Wilcoxon signed-rank test was used to compare the number of conspecific and heterospecific pollen loads of the floral visitors, while the Kruskal-Wallis test was conducted to compare the pollen loads between the visitors. All analyses were conducted using IBM Statistical Package for Social Science (SPSS) version 23.

RESULTS

Diversity of Arthropods Collected from Oil Palm Inflorescence

In total, 1969 arthropod individuals from three classes; Insecta, Arachnida and Diplopoda were collected from the oil palm inflorescences (Figure 3, Table 1). Male inflorescence recorded significantly ($\chi^2=1161.07$, $df=1$, $p<0.001$) higher number of individuals (88%) as compared to

the female inflorescence (12%). However, the total number of taxa recorded between the male and female inflorescence was found to be similar, with three taxa for the male and seven taxa for the female inflorescence ($\chi^2=1.70$, $df=1$, $p>0.05$).

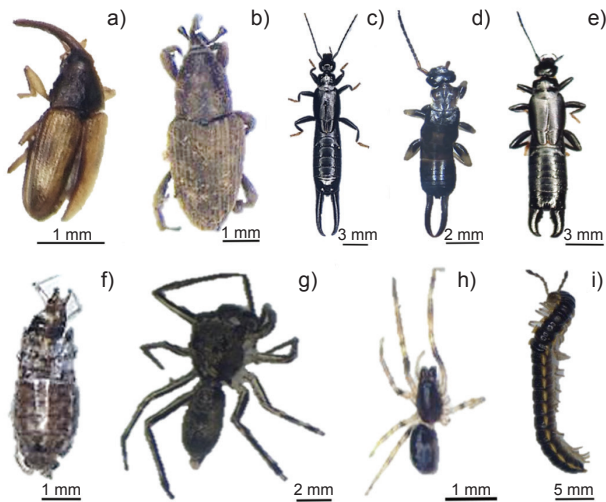


Figure 3. The arthropods collected from the oil palm inflorescence at Ladang Jerangau, TDM Plantation Hulu Terengganu. (a) *Elaeidobius kamerunicus*, (b) Weevil, (c) *Chelisoches morio*, (d) Earwig 1, (e) Earwig 2, (f) Bug (nymph), (g) Spider 1, (h) Spider 2, (i) *Anoplodesmus saussurii*.

For the male flower, *E. kamerunicus* (oil palm weevil) represented 98% of the total individuals collected ($\chi^2=3310.55$, $df=2$, $p<0.001$), while the other individuals were from two unidentified earwig taxa. The Shannon diversity index (H') for these arthropods was 0.09. Meanwhile, the seven arthropod taxa recorded for female inflorescence consisted of four insect taxa from the order Coleoptera (beetles and weevils), Dermaptera (earwigs) and Hemiptera (true bugs), while the other three taxa consisted of two spiders and a millipede. Of these, *Chelisoches morio* (black earwig) significantly recorded the highest number of individuals which represented approximately 61% of the total number of individuals collected from the female inflorescence ($\chi^2=751.89$, $df=6$, $p<0.001$). The Shannon diversity index (H') of the arthropods was 0.82. The Pearson Chi-Square test indicated a significant interaction in the number of arthropods collected and the anthesis stages ($\chi^2=929.13$, $df=2$, $p<0.001$), in which the highest individuals were recorded for the male inflorescence at anthesis ($n=1627$) and the post-anthesis female inflorescence ($n=110$).

For the male inflorescence, a total of 861 larvae were collected from two insect taxa; *E. kamerunicus* and Fungus gnat (Figure 4, Table 2). The majority of the larvae collected were

TABLE 1. NUMBER OF INDIVIDUAL COLLECTED FROM OIL PALM INFLORESCENCES ACCORDING TO ANTHESIS STAGE

Class/Order	Family	Taxa	Male (N=15)				Female (N=15)			
			Pre-anthesis (n=113)	Anthesis (n=1 627)	Post-anthesis (n=1)	Total (n=1 741)	Pre-anthesis (n=36)	Anthesis (n=82)	Post-anthesis (n=110)	Total (n=228)
Insecta/Coleoptera ^a	Curculionidae	<i>Elaeidobius kamerunicus</i>	88	1 623	1	1 712	1	3	1	5
		Weevil	0	0	0	0	1	0	0	1
Insecta/Dermaptera ^b	Chelisochidae	<i>Chelisoches morio</i>	0	0	0	0	31	66	79	176
		Earwig 1	0	4	0	4	0	0	0	0
		Earwig 2	25	0	0	25	0	0	0	0
Insecta/Hemiptera ^c	-	Bug	0	0	0	0	2	3	26	31
Arachnida/Araneae ^d	Salticidae	Spider 1	0	0	0	0	1	4	4	10
	Corinnidae	Spider 2	0	0	0	0	0	1	0	1
Diplopoda/Polydesmida ^e	Paradoxosomatidae	<i>Anoplodesmus saussurii</i>	0	0	0	0	0	5	0	5

Note: ^a - Herbivore (pollen, nectar and tissue feeding); ^b - Carnivore (predator); ^c - Herbivore (plant sap); ^d - Detritivore; ^e - Number of inflorescences observed; n - number of arthropod individuals.

from the post-anthesis inflorescence (83%) as compared to the other two stages ($\chi^2=996.25$, $df=2$, $p<0.001$). From these, 57% emerged into adults *E. kamerunicus* (488 individuals) and Fungus gnat (six individuals).

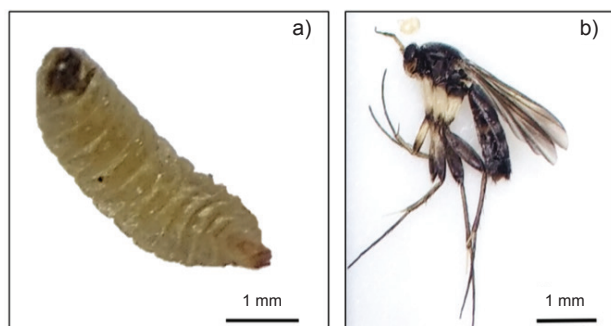


Figure 4. (a) Larvae of *Elaeidobius kamerunicus* and (b) An adult of the Fungus gnat.

Diversity of Insect Visitors to the Oil Palm Inflorescence

A total of 220 insect individuals were trapped while visiting the oil palm inflorescence (Table 3). The female inflorescence recorded significantly ($\chi^2=86.57$, $df=1$, $p<0.001$) lower number of individuals (41 individuals) as compared to the

male inflorescence (179 individuals). Apart from *E. kamerunicus* and Earwig 1 which were recorded earlier, seven additional insect taxa (Figure 5) were captured from two different Orders; five taxa from the Order Hymenoptera and two taxa from the Order Diptera. From the total nine taxa captured, seven and six taxa were recorded for male and female inflorescence respectively, in which only *E. kamerunicus*, Earwig 1, *Dolichogenidea metesae* (braconid wasp) and Fruit fly 1 were recorded for both inflorescences. Not only that, *E. kamerunicus* was also the most common weevil captured which represented 63% of the total captures for both females ($\chi^2=81.32$, $df=5$, $p<0.001$) and males ($\chi^2=456.50$, $df=6$, $p<0.001$) inflorescences. Fruit fly 1 was the second highest capture, in which the captures for male inflorescence (60 individuals) were significantly higher ($\chi^2=52.69$, $df=1$, $p<0.001$) than the captures for the female inflorescence (seven individuals).

Pollen Load of the Arthropods Visiting the Oil Palm Inflorescence

From the total number of individuals trapped, only a single individual of *D. metesae* visiting the female inflorescence and 20 individuals (17 of Fruit fly 1, and three of *E. kamerunicus*) visiting the male inflorescence were negative for pollen load and

TABLE 2. NUMBER OF ADULT INDIVIDUALS EMERGED FROM LARVAE COLLECTED FROM THE OIL PALM INFLORESCENCES FOR EACH ANTHESIS STAGE

Order/ Family	Taxa	Common name	Anthesis stages			
			Pre-anthesis (N=2)	Anthesis (N=143)	Post-anthesis (N=716)	Total (N=861)
Coleoptera/ Curculionidae	<i>Elaeidobius kamerunicus</i>	Oil palm weevil	2	54	432	488
Diptera	-	Fungus gnat	0	4	2	6

Note: N - number of larvae reared.

TABLE 3. THE LIST AND ABUNDANCE OF INSECTS TAXA VISITING THE OIL PALM INFLORESCENCES

Order	Family	Taxa	Number of individuals	
			Male (N=179)	Female (N=41)
Coleoptera ^α	Curculionidae	<i>Elaeidobius kamerunicus</i>	113	26
Dermaptera ^β	-	Earwig 1	2	2
Hymenoptera ^β	Formicidae	<i>Anoplolepis gracilipes</i>	-	3
		<i>Polyrhachis</i> sp.	1	-
	Ichneumonidae	<i>Buysmania oxymora</i>	1	-
	Braconidae	<i>Dolichogenidea metesae</i>	1	2
	-	Wasp	1	-
Diptera ^γ	Drosophilidae	Fruit fly 1	60	7
	-	Fruit fly 2	-	1

Note: ^α - Herbivore (pollen, nectar and tissue feeding); ^β - Carnivore (predator); ^γ - Herbivore (various plant materials); N - Number of visitors.

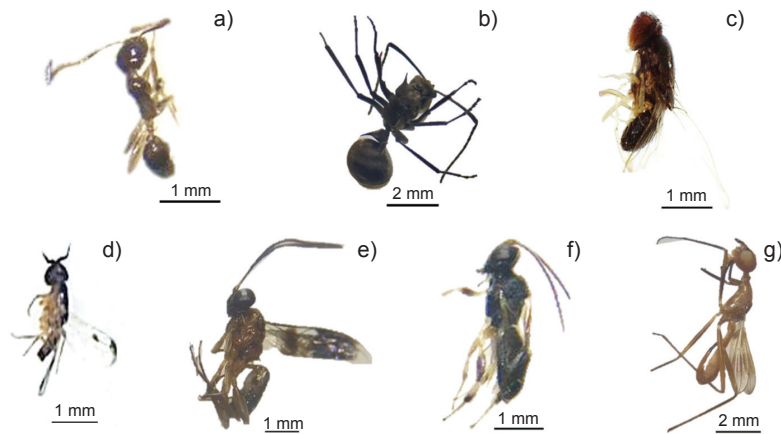


Figure 5. (a) *Anoplolepis gracilipes*, (b) *Polyrhachis* sp., (c) Fruit fly 1, (d) Fruit fly 2, (e) *Buysmania oxymora*, (f) *Dolichogenidea metesae* and (g) Wasp.

TABLE 4. NUMBER (MEAN $\pm$ SE) OF POLLEN GRAINS CARRIED BY THE INSECTS CAPTURED VISITING THE OIL PALM INFLORESCENCE AT LADANG JERANGAU TDM PLANTATION

Taxa	Male inflorescence		Female inflorescence		
	Conspecific	N	Conspecific	Heterospecific	N
<i>Elaeidobius kamerunicus</i> ***	36.05 $\pm$ 13.98 ^{ab}	110	13.31 $\pm$ 6.99	0.54 $\pm$ 0.30	26
Earwig 1*	7.50 $\pm$ 5.00 ^{bc}	2	5.00 $\pm$ 5.00	2.00 $\pm$ 0.00	2
<i>Anoplolepis gracilipes</i>	-	-	2.50 $\pm$ 0.50	0.00 $\pm$ 0.00	2
<i>Polyrhachis</i> sp.	15.00 $\pm$ 0.00	1	-	-	-
<i>Buysmania oxymora</i>	2.00 $\pm$ 0.00	1	-	-	-
<i>Dolichogenidea metesae</i>	89.00 $\pm$ 0.00	1	3.50 $\pm$ 1.50	0.00 $\pm$ 0.00	2
Wasp	1.00 $\pm$ 0.00	1	-	-	-
Fruit fly 1**	7.40 $\pm$ 2.02 ^c	43	8.86 $\pm$ 3.54	0.29 $\pm$ 0.29	7
Fruit fly 2	-	-	2.00 $\pm$ 0.00	0.00 $\pm$ 0.00	1

Note: For visitors to the male inflorescence, the different lowercase letter indicates a significant difference in the mean number of conspecific pollen loads between *E. kamerunicus*, Earwig 1 and Fruit fly 1 from multiple comparisons (stepwise step-down method) following significant result at $p < 0.001$ of the Kruskal-Wallis test. For visitors to the female inflorescence with conspecific and heterospecific pollen loads, the Wilcoxon signed-rank test conducted indicates: *no significant difference, **significant at $p < 0.05$ and ***significant at $p < 0.001$ in the number of grains between the two pollen types; N = Number of individuals observed.

thus were excluded from further analyses. A total of 159 insects visited the male inflorescence carrying only the conspecific grains (Table 4). Of these, an individual of *D. metesae* recorded the highest number of grains (89 grains), while *E. kamerunicus* recorded the second highest number (mean $\pm$ SE) of pollen load with 36.05 $\pm$ 13.98 grains. Further analysis revealed that the mean number of pollen loads carried by *E. kamerunicus*, Earwig 1 and Fruit fly 1 were significantly different (Kruskal-Wallis test, $H = 17.97$, $df = 2$, $p < 0.001$).

Four pollen types were recorded from the 40 individuals visiting the female inflorescence which consisted of the conspecific pollen and three unidentified types (the heterospecific pollen). Insect visitors from the six taxa captured visiting the female inflorescence were found with conspecific pollen loads, in which *E. kamerunicus* carried the highest number of grains (13.31 $\pm$ 6.99). Further analysis showed that the mean number of pollen

loads carried by individuals across the six taxa was similar (Kruskal-Wallis test, $H = 3.131$, $df = 5$, $p = 0.680$). In addition, *Anoplolepis gracilipes* (yellow crazy ant), *D. metesae* and Fruit fly 2 only carried the conspecific pollen grains on their bodies.

In contrast, insects from only three taxa (*E. kamerunicus*, Fruit fly 1 and Earwig 1) that visited the female inflorescence were found carrying the heterospecific pollen loads. Earwig 1 carried a significantly higher number of grains (2.00 $\pm$ 0.00) as compared to the other two taxa (Kruskal-Wallis test, $H = 7.055$, $df = 2$, $p = 0.029$). Only *E. kamerunicus* (Wilcoxon signed-rank test, $z = 4.475$, $p < 0.001$) and Fruit fly 1 (Wilcoxon signed-rank test, $z = 2.735$, $p = 0.018$) however were found to show significantly higher conspecific rather than heterospecific pollen loads. While for Earwig 1, the conspecific and heterospecific pollen load was not significantly different (Wilcoxon signed-rank test, $z = 0.447$, $p = 0.655$). Between the three taxa that visited both

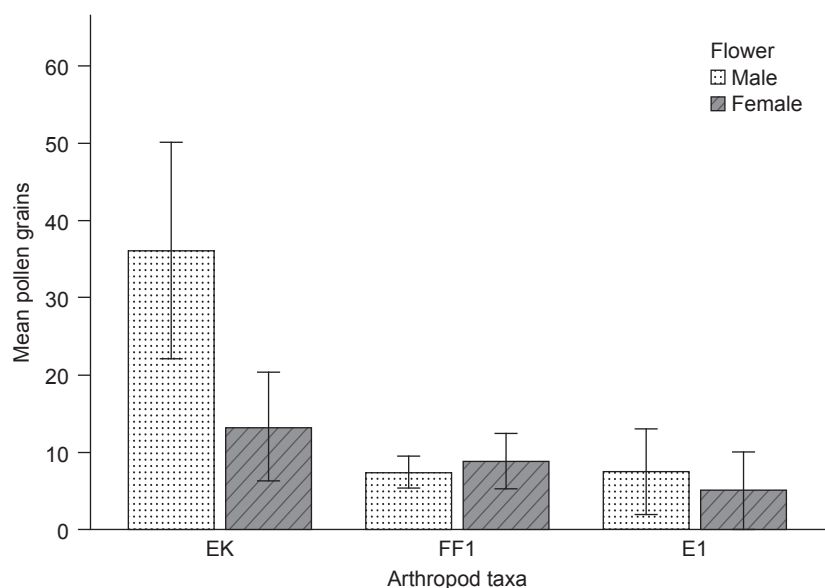


Figure 6. Mean of the conspecific pollen load for the three insects that visited both the male and female oil palm inflorescence. Error bars indicate a 95% confidence interval. EK = *Elaeidobius kamerunicus* (male inflorescence = 110 individuals and female inflorescence = 26 individuals), FF1 = Fruit fly 1 (male inflorescence = 43 individuals and female inflorescence = 7 individuals), E1 = Earwig 1 (male inflorescence = 2 individuals and female inflorescence = 2 individuals).

male and female inflorescences (*D. metesae* was excluded for this comparison because only an individual was caught at the male inflorescence), only *E. kamerunicus* carried a significantly higher number of conspecific pollen grains (Mann-Whitney test, $U=792.500$, $p<0.001$) while visiting the male than the female inflorescence (Figure 6).

DISCUSSION

The present study reported a total of 14 insect taxa, two spiders and a millipede from the oil palm inflorescence (Tables 1, 2 and 3). From these, nine taxa were recorded for male inflorescence while 12 taxa were recorded for female inflorescence. Although male inflorescence recorded a higher abundance of arthropods, the species richness however was low (only four taxa including a fly taxon at the larval stage), although trappings yielded higher richness (seven taxa). Female inflorescence, in contrast, recorded an almost equal arthropod richness with seven and six taxa from collected and trapped individuals, respectively. However, the arthropod collected at this flower was with much lesser abundance as compared to the male inflorescence. The higher abundance and richness of the insect visitors to the male oil palm inflorescence as compared to the female inflorescence (Egonyu *et al.*, 2021) was due to the strong anise-seed scent emitted by the inflorescence which attracted the insect visitors (Anggraeni *et al.*, 2013; Lajis *et al.*, 1985), as well as the availability of pollen grains as a food source (Sambathkumar and Ranjith, 2011; 2015; Syed, 1979).

Furthermore, the anthesis phase of the male inflorescence was also over a longer period than the female (Forero *et al.*, 2012); between 6-8 days for the male as compared to 3-5 days for the female observed at the study site, thus the food reward (pollen grains) was offered for a longer period for the visitors. Female inflorescence, on the other hand, emits a similar scent to attract visitors (Lajis *et al.*, 1985) but due to a lack of food sources, the insects stay on the inflorescence only briefly (Syed, 1979). Hence the availability of food and the scent emitted by the inflorescence regulate the movement of these visitors from male to female inflorescence (Anggraeni *et al.*, 2013; Sambathkumar and Ranjith, 2011). While at the male inflorescence, these visitors usually collected the pollen grains on their bodies, thus likely transferring the grains to the stigma while visiting the female inflorescence later to initiate pollination.

A higher diversity of insects in oil palm plantations was reported in Malaysia (*e.g.*, Siti Khairiyah *et al.*, 2013) and elsewhere (*e.g.*, Siregar *et al.*, 2016 in Indonesia), and insects collected specifically at the oil palm inflorescence (*i.e.*, the flower visitors) were usually associated with their role as pollinators. The number of insect taxa recorded in the present study indeed was very much lower as compared to more intensive documentation of the flower visitors such as by Rizali *et al.* (2019) and Egonyu *et al.* (2021) in Indonesia and Uganda, respectively. In contrast, Bazurto *et al.* (2018) despite conducting a year of trappings of oil palm insect pollinators from more than 800 palm trees in Colombia, they reported only two insect taxa. The diversity of insect visitors

in the oil palm plantation was noted to be higher in the areas closer to natural habitat than the areas farther away (Egonyu *et al.*, 2021), although earlier reports indicated no relation in the diversity of insect pollinators in oil palm plantations and its distance from the forest habitat (Mayfield, 2005). A high abundance of oil palm inflorescence also might attract the insects to forage in the plantation areas (Siregar *et al.*, 2016).

Elaeidobius kamerunicus was the most predominant arthropod recorded for male inflorescence, in which adults were predominantly collected from the anthesising inflorescences, while its larvae were extracted from the post-anthesis inflorescence (Table 2). Moreover, a high abundance of this weevil was also captured at the female inflorescence, carrying high conspecific pollen grains on their bodies (Table 4 and Figure 6). This weevil thus undoubtedly is the principal pollinator for the oil palm trees (Auffray *et al.*, 2017; Kouakou *et al.*, 2014; Melendez-Jacome *et al.*, 2019; Sambathkumar and Ranjith, 2015; Syed, 1979) even in the tropical Asian region where it was introduced (Li *et al.*, 2019; Syed *et al.*, 1982; Yue *et al.*, 2015). Mutualism between the oil palm trees and this weevil is further explained by the importance of male inflorescence for the weevil to complete its life cycle (Adaigbe *et al.*, 2011).

The presence of predatory insects such as ants, wasps and earwigs, together with the two spider taxa recorded at the inflorescence, indicates potential predators of the insect pollinators (Bos *et al.*, 2008; Muhammad Luqman *et al.*, 2017; Panabang *et al.*, 2017). A high abundance of these predatory arthropods; Earwig 2 at the pre-anthesis male inflorescence, *C. morio* at the anthesis and post-anthesis female inflorescence, and particularly the spiders which were only collected from the female inflorescence, is certainly a concern. Predation risk exists not only to the adult individuals but predation on larvae by the Earwig 1 was also documented (Figure 7) during the cataloguing of the arthropod samples. While Earwig 1 was noted as a predator of larvae, surprisingly it was also observed to carry the conspecific grains while visiting both male and female inflorescences and was thus also likely a pollinator (Table 4 and Figure 6). Although no further investigation was made on the effect of these predatory arthropods on the pollination success of the oil palm trees in the study area, predation on larvae and adult *E. kamerunicus* might impose negative results on the fruit yield from its population reduction (Li *et al.*, 2019; Muhammad Luqman *et al.*, 2017).

Arthropod predators nevertheless are important natural biological control agents in oil palm plantations in Malaysia (Denan *et al.*, 2020). For example, *C. morio* was found to predate heavily on the larvae of the coconut spike moth (*Tirathaba*

ruvifena), one of the important insect pests of the oil palm tree (Zhong *et al.*, 2016). Other than that, the presence of the parasitic wasp, *D. metesea*, together with the parasitoid wasp, *Buysmania oxymora*, suggested natural pest controlling activities at the study area particularly aimed at the other prominent pest of the oil palm tree, the bagworm, *Metisa plana* (Halim *et al.*, 2017; Kamarudin and Arshad, 2016). Hence maintaining these arthropods in the oil palm plantation could minimise the use of pesticides for pest control, thus supporting sustainable agricultural practices.



Figure 7. Predation on larvae by Earwig 1 was observed while conducting sample cataloguing.

A total of three fly taxa were noted in the study area, in which larvae of a single taxon were collected from the post-anthesis male inflorescence (Figure 4 and Table 2), indicating this inflorescence acted as a breeding site of the fly. For the coleopteran and dipteran insect pollinators, it is common for the larvae to develop in the decomposed male inflorescence, in which the insects collected pollen grains on their bodies during oviposition at the anthesising inflorescence (Sakai, 2002). For the dipteran flies associated with the oil palm inflorescence, however, their contribution to pollination of the oil palm trees is still not fully understood (Mayfield, 2005; Rizali *et al.*, 2019; Sambathkumar and Ranjith, 2011). In the present study, the Fruit fly 1 which was captured at a notably higher abundance at the male inflorescence (Table 4 and Figure 6), was found with high conspecific pollen loads on their bodies, thus showing its importance as pollen vectors for the oil palm trees. Not only that, Fruit fly 2, together with *A. gracilipes* and *D. metesae*, were also found with conspecific pollen grains on their bodies while visiting the female inflorescence (Table 4), indicating their potential to contribute to the pollination of the inflorescence. Unfortunately, *A. gracilipes* was known to be an invasive ant and predate on other ant species in agroecosystem habitats (Bos *et al.*, 2008; Sinu *et al.*, 2017).

One of the other noteworthy results in the present study was the record of black earwig *C. morio* collected from the oil palm inflorescence, which is a new locality report for this species in Peninsular Malaysia. This species was previously recorded only from Selangor, Kuala Lumpur and Penang Island (Kamimura *et al.*, 2016a). In Penang Island, earwig fauna was reported to occupy almost all environmental conditions and vegetation types, including plantations. Another interesting finding was five individuals of a polydesmid millipede, *Anoplodesmus saussurii*, which were collected at the anthesising female inflorescence (Table 1). Millipedes, in general, are detritivores which consume decaying plant materials and have never been reported to feed on living plants. Millipedes are usually found under the decaying litter layers (Decker and Tertilt, 2012), or in the soil, as reported by Sakiah *et al.* (2017) and Jhon *et al.* (2019) in oil palm plantations, which help in the mixing of the decomposing materials into the soil layers. As for *A. saussurii*, it was reported to be endemic to Sri Lanka (De Zoysa *et al.*, 2016), but potentially is more widespread with more deep observations being made with more published documentation available (Decker and Tertilt, 2012; Golovatch and Stoev, 2013). In Singapore, this species is commonly found aggregated in a large numbers (Decker and Tertilt, 2012), hence the few individuals collected from the inflorescence in the present study could be accidental. Information on the taxonomy, distribution and ecology of the earwigs (Kamimura *et al.*, 2016a; 2016b) and millipedes (Golovatch and Stoev, 2013; Likhitrakarn *et al.*, 2016; Moseley, 2006) in Peninsular Malaysia is currently limited, thus the observations reported in this study are valuable to add more knowledge of these inconspicuous arthropod faunas.

The present study documented a high diversity of arthropods in the oil palm plantation, indicating that this invertebrate fauna is an important component in this agricultural area. Arthropod fauna, particularly the insects, provides important ecosystem services such as pollination, biological control (through predation, herbivory and parasitism), food provisioning and decomposition of organic matters, although these could be under-represented (Noriega *et al.*, 2018). In agricultural ecosystems, knowledge of insect pollinators is biased towards charismatic taxa such as the lepidopteran, hymenopteran and coleopteran insects, while the functional roles provided by other insects are still not fully understood (Hortal *et al.*, 2015). Results in our study, for example, revealed that several predatory insects have the potential to act as pollinators for the oil palm trees. Moreover, although several herbivorous insects were recorded, we did not find any evidence to suggest that these insects caused a negative impact

on the inflorescences such as damage to the floral tissues. We are aware that this observation is based on small sample size, but we believe that the findings from this study contribute significantly to the current knowledge of these important insect communities in the agricultural area. This may lead to better agricultural and management practices that support greater biodiversity in this important ecosystem.

CONCLUSION

Arthropod fauna of the oil palm inflorescence at Ladang Jerangau Terengganu consisted of 17 taxa, with insects (14 taxa) being the most predominant fauna recorded. Other than to feed on the floral source (pollen) of the oil palm inflorescence, several insect taxa were potentially preying on other insects at the inflorescence. Apart from *E. kamerunicus*, an unidentified fly taxon was also observed to oviposit at the male inflorescence, and predation on the larvae of these two insects by the earwig was witnessed in this study. The palm weevil, *E. kamerunicus* was again detected as the main pollinator of the oil palm tree, by collecting the pollen grains on their bodies while aggregating at the male inflorescence, followed by transporting the conspecific grains to the female inflorescence. In addition, the potential role of other insect taxa as pollinators for the oil palm trees were also detected from their conspecific pollen loads while visiting the male inflorescence (*i.e.*, an unidentified earwig, *A. gracilipes*, *D. metesae* and 2 unidentified flies), as well as while visiting the female inflorescence (*i.e.*, *Polyrhachis* sp., *B. oxymora* and *D. metesea*). This study also reported the first record of *C. morio* in Terengganu, which is also a new locality record of this earwig taxon in Peninsular Malaysia. We suggest that more detailed studies be conducted on these elusive insects, in order to increase the current understanding of their ecological roles in oil palm plantations.

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OIL PALM (*Elaeis guineensis*) SEED CHARACTERISTICS AND GERMINATION POTENTIAL AS INFLUENCED BY MATURITY STAGE AND FRUITLET POSITION ON A BUNCH

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ABSTRACT

Oil palm is mainly propagated through dura × pisifera hybrid (DxP) seeds. In the natural environment, oil palm seeds require more than eight months of storage to achieve 25% germination due to a combination of morphological and physiological dormancy. Therefore, improvement in terms of the percentage of germination, uniformity and reduction in time to germination will benefit seed producers tremendously. In this study, the effect of harvesting at three maturity stages (18, 20 and 22 weeks after pollination or WAP) and six seed positions within a bunch (Proximal-Base, Proximal-Apex, Middle-Base, Middle-Apex, Distal-Apex and Distal-Base) on seed characteristics and germination potential were evaluated in a split-plot design. Seeds harvested at 20 WAP had 80% black-coloured seeds with the highest germination of 85.1%, compared with 70.5% and 80.9% at 18 and 22 WAP, respectively. Increasing seed maturity from 18 to 20 WAP showed more seeds shifting from semi-white and white to black (up to 40% more). Seeds located at the base region of the spikelet were smaller in size and were predominantly white with no differences in germination capacity.

Keywords: fruitlet position, maturity stage, oil palm, seed germination.

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INTRODUCTION

Seed maturity is an important element in determining the optimal harvesting time to produce high-quality seeds. Studies have shown the importance of seed maturity stages in the development of seed characteristics such as seed size, dry weight, the biochemical composition, including abscisic acid and gibberellin levels (Tetteh *et al.*, 2018; Vishal *et al.*, 2018), as well as the germination capacity (Lima *et al.*, 2012; Samarah and Mullen, 2004). In DxP oil palm seed production, the harvesting can generally

be done between 18 to 22 weeks after assisted pollination, according to the standard commercial practice (SDSAS, 2020). In practice, the 20 weeks after the pollination stage is perceived as the most practical for harvesting, with almost all the fruitlets still attached to the spikelets. However, germination is reported to range from 62.2% to 72.0%, with sporadic germination spread over 60 days (SDSAS, 2018). The oil palm seeds requirement in Malaysia stands at 50 million seeds per year in 2021, selling at an average of RM2.00 per germinated seed (MPOB, 2022). Hence, the relatively low (~68%) and non-uniform germination cause a tremendous loss to the industry. A detailed study on biochemical changes and histology of the oil palm seed development indicates that seed development is completed 18 weeks after anthesis (Kok *et al.*, 2015), raising the question of the advantage of early harvesting without affecting the oil palm seed quality. In oil palm, early harvesting has been carried out occasionally due to high demand from the nursery

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and seed buyer, however, the germination capacity has not been documented. Another aspect of seed development that contributes to a lower number of saleable seeds is the endocarp colour. Upon the depericarp process, around 20% of seeds are generally discarded due to their white colour, which was perceived as having low quality. A recent study by Norsazwan *et al.* (2022) indicated that seed endocarp colour was not associated with abnormality and lower quality, yet the issue of perception from potential customers remained.

Generally, an oil palm fruit bunch consist of 1000 to 3000 fruitlets, which develop from a single inflorescence (Corley and Tinker, 2015). Although assisted pollination is conducted thoroughly to ensure all the anthesising female florets receive the intended *pisifera* pollen at the same time, variation may persist due to the differences from florets that are located at different sections within the bunch. Baydar and Erbaş (2005) reported that sunflower seed development was dependent on the seed position within the flower head. Seeds that were located at the outer side of the head were found to have higher dry weight with increased levels of palmitic, stearic and oleic acids. In addition, variation in seed characteristics was associated with spatial differences which influence the photosynthetic supply to the developing seeds (Bareke, 2018). In commercial seed production, the oil palm bunch is currently processed as a whole; without separating the seeds into different sections. While this is a practical system, it could also account for the variation in seed morphology, the asynchronous germination capacity and the incidence of white seeds. Therefore, this study aims to evaluate the effect of seeds harvested at different maturity stages on seed characteristics and germination. In addition, the characteristics of seeds located at different positions within a fruit bunch on germination and quality will be established.

MATERIALS AND METHODS

Seed Source

Oil palm D×P CALIX 600 seeds used in this study were obtained from Sime Darby Seeds and Agricultural Services (SDSAS). The *Dura* mother palm from a similar genetic lineage (code: BM 2515 to BM 2525) was selected from field PT 100 Ladang Dusun Durian, Sime Darby Plantations, Banting, Selangor (2°48'16.2"N 101°27'21.2"E). *Pisifera* pollen source (code: BM 2323/34) was used in controlled pollination. The procedure for controlled pollination of D×P seeds was conducted following the MS157: 2017 guidelines (Department of Standards, 2017). The harvested bunch was immediately transported to the seed processing facility of Sime Darby

Seeds and Agricultural Services (SDSAS), Banting, Selangor, Malaysia.

Experimental Design

The experimental design used was a split-plot, with the seed maturity stage (as the main plot) and the seed position (as the sub-plot). Three levels of seed maturity stages (18 WAP, 20 WAP and 22 WAP) and six positions on the bunch (Proximal-Base, Proximal-Apex, Middle-Base, Middle-Apex, Distal-Apex and Distal-Base) were evaluated. All the treatment combinations were replicated four times by using four different oil palm fruit bunches from similar CALIX 600 materials. The oil palm bunch was initially divided into three equidistance sections from the stalk and recorded as proximal, middle and distal. Each of the sections was further divided into the base and apex region of the spikelet (*Figure 1*).

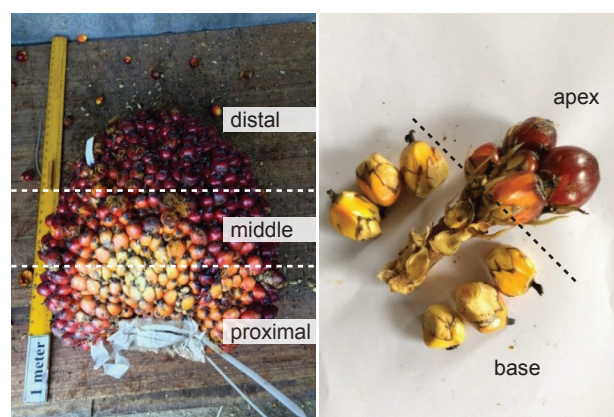


Figure 1. D×P bunch was divided and separated into three equal sections (distal, middle and proximal) (left) Spikelets from each of the sections were further divided into apex or base (right).

The detached fruitlets were then placed inside a mesocarp removal (also known as depericarper) machine. After the mesocarp removal, the seeds were immersed and continuously agitated with 0.05% Benomyl 50% WP (95% a.i) solution for 5 min to prevent fungal infection.

Seed Endocarp Colour and Physical Characteristics

Four replicates of 100 seeds from each treatment were randomly placed inside a plastic container for seed endocarp colour assessment. The percentage of black (endocarp surface is black or dark brown, with <10% white), semi-white (10%-90% endocarp surface is white coloured) and white (endocarp surface is white, with <10% black) seeds on each position was recorded as described by Norsazwan *et al.* (2022). Next, four replicates of 10 seeds were randomly selected for seed physical characteristics evaluation. The seed dry weight (g), shell thickness (mm), seed length/width (mm), kernel length

(mm), embryo length/width as well as operculum diameter (mm) were measured. For embryo measurement, a steel clamp was used to cut open the seed and expose the endosperm region, without damaging the embryo located directly underneath the fibre plug area. The embryo was then carefully excised from the seed using a scalpel blade. Embryo length and width (mm) were measured using a stereo microscope attached to a digital camera (Leica model EZ4D).

Seed Viability

Seed viability was measured using both tetrazolium (TZ) and germination tests to evaluate the germination potential and actual germination capacity of the dormant seed. Four replicates of 10 embryos were excised and soaked in distilled water for 3 hr. It was then transferred into a vial containing 5 mL of 1.0% (w/v) Tetrazolium chloride solution. The vials were kept in darkness for 17 hr prior to observation. The staining was categorised as red (viable), pink (non-viable) and white (non-viable). The mean percentage of the viable embryo was recorded. Prior to the germination test, seeds were subjected to heat treatment of $40^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 60 days. After that, the treated seeds were imbibed in water for 10 days, separated into 15×10 cm plastic bags, containing four replicates of 100 seeds and placed inside a germination room at $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$ germination temperature. Germinated seeds were monitored daily by recording the number of seeds showing normal emergence of radicles from the fibre plug according to the Sime Darby Seed Production Unit (SPU) standard evaluation procedures. Germinated seeds were removed from the bag and the remaining non-germinated seeds were kept until the end of the germination test period (day 60). The percentage germination was determined as a percentage at time intervals over the course of the germination period, using the following Equation (1):

$$\text{Germination (\%)} = \frac{\text{Total number of seeds germinated}}{\text{Number of seeds used}} \times 100 \quad (1)$$

Statistical Analysis

Analysis of variance (ANOVA) was performed by Microsoft Excel and Statistical Analysis Software, SAS 9.4 (SAS Institute, Cary, NC). A significance level of $p \leq 0.05$ was used for Duncan's Multiple Range Test (DMRT) throughout this study.

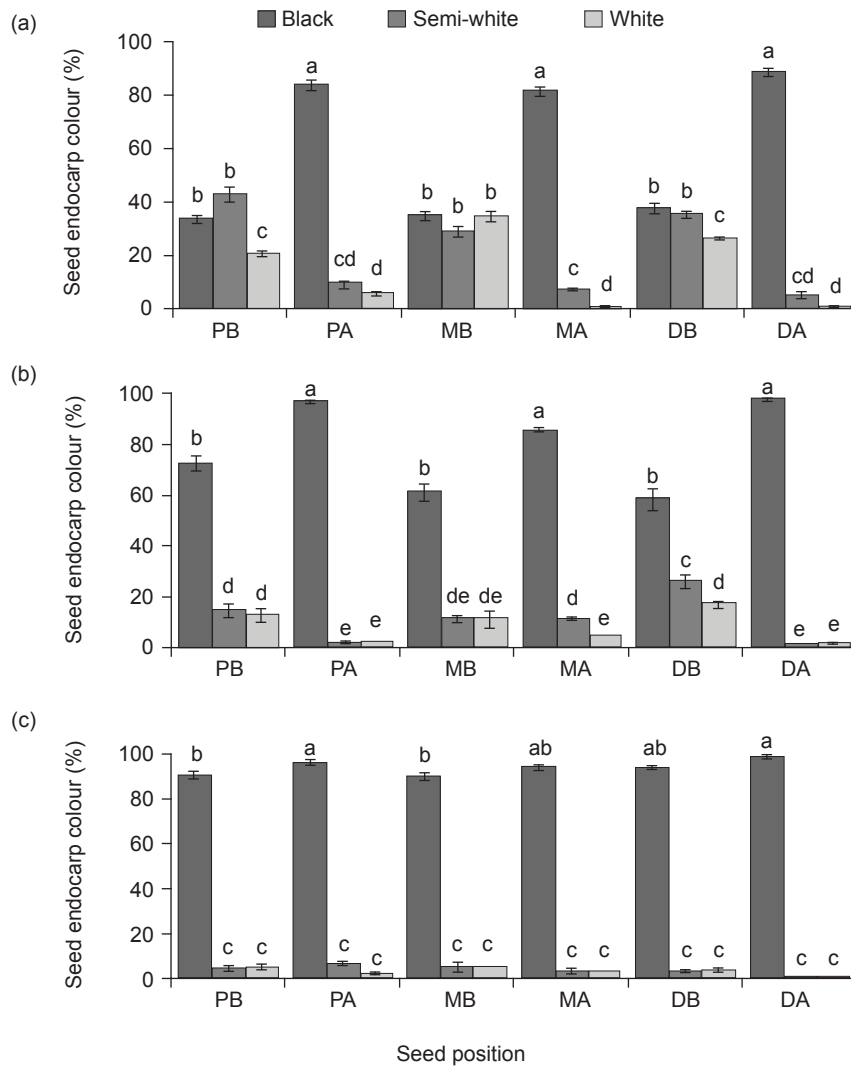
RESULTS AND DISCUSSION

The unique structure of oil palm inflorescence, which consisted of up to 3000 seeds, formed along

hundreds of spikelets tightly attached to the stalk (Corley and Tinker, 2015) was found to influence the fruitlets and seed morphological characteristics. In this study, it was recorded that as the maturity stages increased from 18 to 22 WAP, there was a clear shift in seed colour, from mostly white and semi-white to black. The percentage of black seeds increased significantly, while semi-white and white seeds decreased from 18 to 22 WAP, for every section of the oil palm fruit bunch. For instance, the proximal-base region had only 33.75% of black seeds at 18 WAP and this increased to 72.58% and 90.50% at 20 WAP and 22 WAP, respectively (Figures 2 and 3).

Seeds that developed closer to the stalk (the base part of the spikelet), regardless of any position on the bunch (either proximal, middle or distal) had higher percentages of white seeds in comparison to the respective apex section of the spikelet. At 18 WAP, the proximal region of the bunch; proximal-base, recorded 33.75% of black, 46.00% semi-white and 20.25% of white seeds. On the other hand, the proximal apex was composed of 84.00% black, 10.00% semi-white and 6.00% white seeds. Similarly, the middle and distal sections had 20%-30% more semi-white and white seeds at the base, in comparison with the apex section of the spikelet.

However, as the harvesting was delayed, the differences between white and semi-white seeds between the proximal and apex of the spikelet decreased significantly, as can be seen at 22 WAP. Seeds located at the base region of the spikelet experience less exposure to sunlight, thus reducing pigmentation which resulted in lighter-coloured seeds (Norsazwan *et al.*, 2022). The pigmentation in seeds is known to be mainly caused by the accumulation of flavonoids, which is influenced by sunlight exposure (Marles *et al.*, 2003). Higher light intensities were reported to increase flavonoid content as a protective measure from harmful UV radiation (Su *et al.*, 2006; Xie and Wang, 2006). These include anthocyanins (red and purple), flavonols (pale yellow to colourless) and tannins (colourless, which later turn brown with oxidation) (Wan *et al.*, 2016; Yu, 2013). Earlier studies from Marles *et al.* (2003) had reported that dark brown coloured seed coat in *Brassica carinata* was mainly due to the presence of condensed tannins (proanthocyanidins) and the absence of anthocyanin. In terms of seed dry weight, several distinct differences were observed between the base and apex within the spikelet, for all three seed maturity stages. Seeds located at the apex region consistently showed higher seed weight. For example, at 18 WAP, proximal-apex, middle-apex and distal-apex recorded 3.79, 3.80 and 3.88 g per seed, respectively (Table 1).



Note: PB - proximal-base; PA - proximal-apex; MB - middle-base; MA - middle-apex; DB - distal-base; DA - distal-apex. Different letters indicate significant differences within each column, at a 5% probability level.

Figure 2. Mean percentage of black, semi-white and white seeds at different sections of the oil palm bunch harvested at (a) 18, (b) 20, and (c) 22 weeks after pollination (WAP).

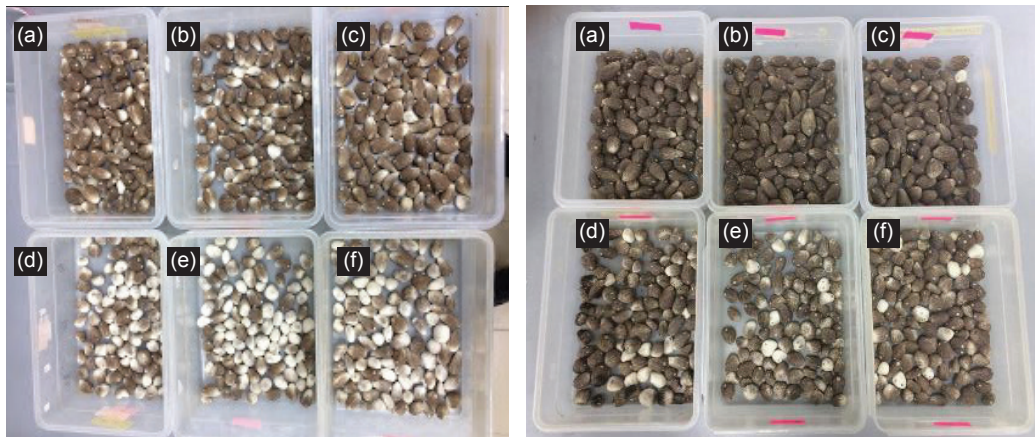


Figure 3. Seed endocarp colour changes from 18 WAP (left) to 22 WAP (right). (a) Proximal-apex, (b) middle-apex, (c) distal-apex, (d) proximal-base, (e) middle-base and (f) distal-base.

TABLE 1. SEED CHARACTERISTICS FOR DIFFERENT SEED MATURITY STAGES AND SEED SECTIONS

Seed stage	Seed position	Mean seed dry weight (g)	Mean seed length (mm)	Mean seed width (mm)	Mean operculum diameter (mm)	Mean shell thickness (mm)
18	PB	2.38 ^g	17.08 ^{de}	17.49 ^a	3.62 ^{ab}	3.17 ^{ab}
	PA	3.79 ^{a-c}	26.32 ^a	17.83 ^a	3.50 ^{a-c}	3.18 ^{ab}
	MB	2.97 ^{d-f}	14.27 ^e	17.95 ^a	3.31 ^{c-e}	3.01 ^{bc}
	MA	3.80 ^{a-c}	26.47 ^a	16.98 ^a	3.02 ^f	2.45 ^g
	DB	3.36 ^{b-d}	16.49 ^{de}	17.07 ^a	3.39 ^{a-d}	2.72 ^{d-f}
	DA	3.88 ^{a-c}	25.06 ^{ab}	13.10 ^b	3.07 ^{ef}	2.96 ^{b-d}
20	PB	2.79 ^{e-g}	15.71 ^{de}	16.84 ^a	3.44 ^{a-d}	3.08 ^{a-c}
	PA	3.87 ^{a-c}	24.82 ^{ab}	17.31 ^a	3.44 ^{a-d}	3.03 ^{a-c}
	MB	3.56 ^{bc}	18.16 ^d	16.71 ^a	3.35 ^{b-d}	2.99 ^{b-d}
	MA	3.47 ^{b-d}	25.76 ^{ab}	16.84 ^a	3.00 ^f	2.50 ^{fg}
	DB	2.70 ^{fg}	14.51 ^e	16.43 ^a	3.31 ^{c-e}	2.85 ^{c-e}
	DA	3.67 ^{a-c}	23.38 ^{bc}	13.72 ^b	3.24 ^{d-f}	3.08 ^{a-c}
22	PB	2.91 ^{d-g}	16.81 ^{de}	17.15 ^a	3.35 ^{cd}	3.00 ^{b-d}
	PA	4.23 ^a	23.32 ^{bc}	17.97 ^a	3.63 ^a	3.30 ^a
	MB	3.41 ^{b-d}	21.75 ^c	16.52 ^a	3.06 ^{ef}	2.51 ^{fg}
	MA	4.23 ^a	21.58 ^c	17.92 ^a	3.50 ^{a-c}	3.10 ^{a-c}
	DB	3.31 ^{c-e}	16.33 ^{de}	16.00 ^a	3.25 ^{d-f}	2.70 ^{e-g}
	DA	3.95 ^{ab}	18.28 ^d	13.25 ^b	3.25 ^{d-f}	3.02 ^{bc}

Note: PB - proximal-base; PA - proximal-apex; MB - middle-base; MA - middle-apex; DB - distal-base; DA - distal-apex. Different letters indicate significant differences within each column, at a 5% probability level.

In contrast, the proximal, middle and distal-base sections had 2.38 g (proximal), 2.97 g and 3.36 g, respectively. Similar trends were observed for the seeds harvested at 20 and 22 WAP. A similar seed length trend was observed at 18 and 20 WAP. The highest seed lengths were obtained at proximal-apex, middle-apex and distal-apex sections, with values ranging from 23.38 mm to 26.47 mm. The base region of the same spikelet, consistently showed lower seed length values, ranging from 14.20 mm to 18.16 mm. On the other hand, at 22 WAP, only the proximal and distal-apex regions had recorded significantly higher seed lengths, in contrast to the respective base regions. The middle-apex and middle-base showed no significant differences in length. The seed width showed less overall variation as compared to length. However, no specific trend was observed. The seed width ranged from 13.10 mm to 17.98 mm and varied throughout the different seed maturity stages. Interestingly, the operculum size was found to have a weak correlation with the seed weight (Pearson's Correlation Coefficients = 0.11073, p -value <0.05). The mean value ranged from 3.00 mm to 3.63 mm in diameter. In general, oil palm seeds did not show changes in shell thickness from 18 to 22 WAP and the values varied greatly among

different seed positions. The shell thickness was significantly correlated to the operculum diameter (Pearson's Correlation Coefficients = 0.3097). The value ranged from 2.45 mm to 3.30 mm. The proximal sections (for both base and apex) of the bunch consistently showed more than 3.00 mm shell thickness.

Generally, all seeds that were located at the base region of the spikelets showed a more rounded appearance due to the lower length-to-width ratio. In contrast, higher length-to-width ratio of seeds at the apex section resulted in a more elongated morphology. The effect of space competition towards the seed morphological characteristics had been reported in other oilseed species, including sunflower (Gupta *et al.*, 2009), mustard (Munshi and Kochar, 1994; Munshi and Kumari, 1994) and soybean seeds (Guleria *et al.*, 2008). The seeds that were in the central region of the whorl showed the significantly lower size and dry weight in comparison to the outer region (Gupta *et al.*, 2009). In the case of oil palm development, fruitlets located closer to the stalk will experience more spatial restriction due to the bunch position in between two fronds, thus reducing sunlight exposure (pigmentation), along with smaller, rounded fruitlets and seeds (Figure 4).

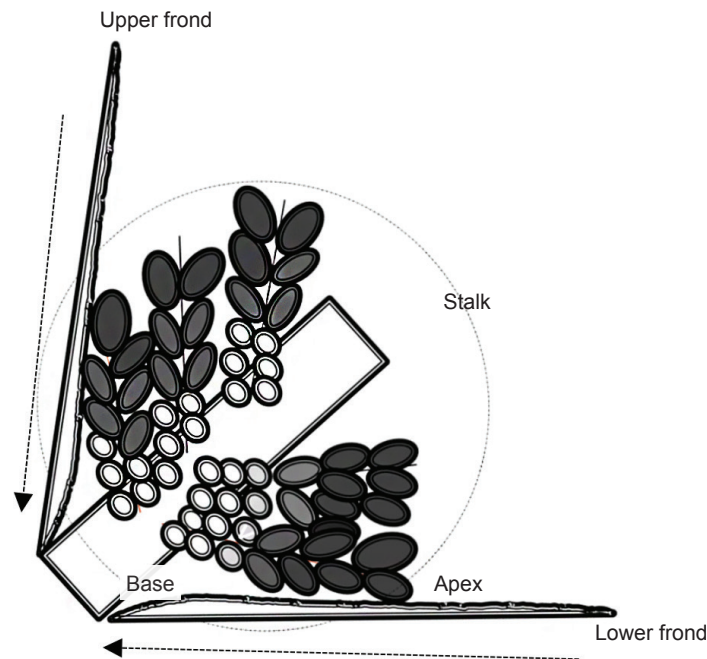
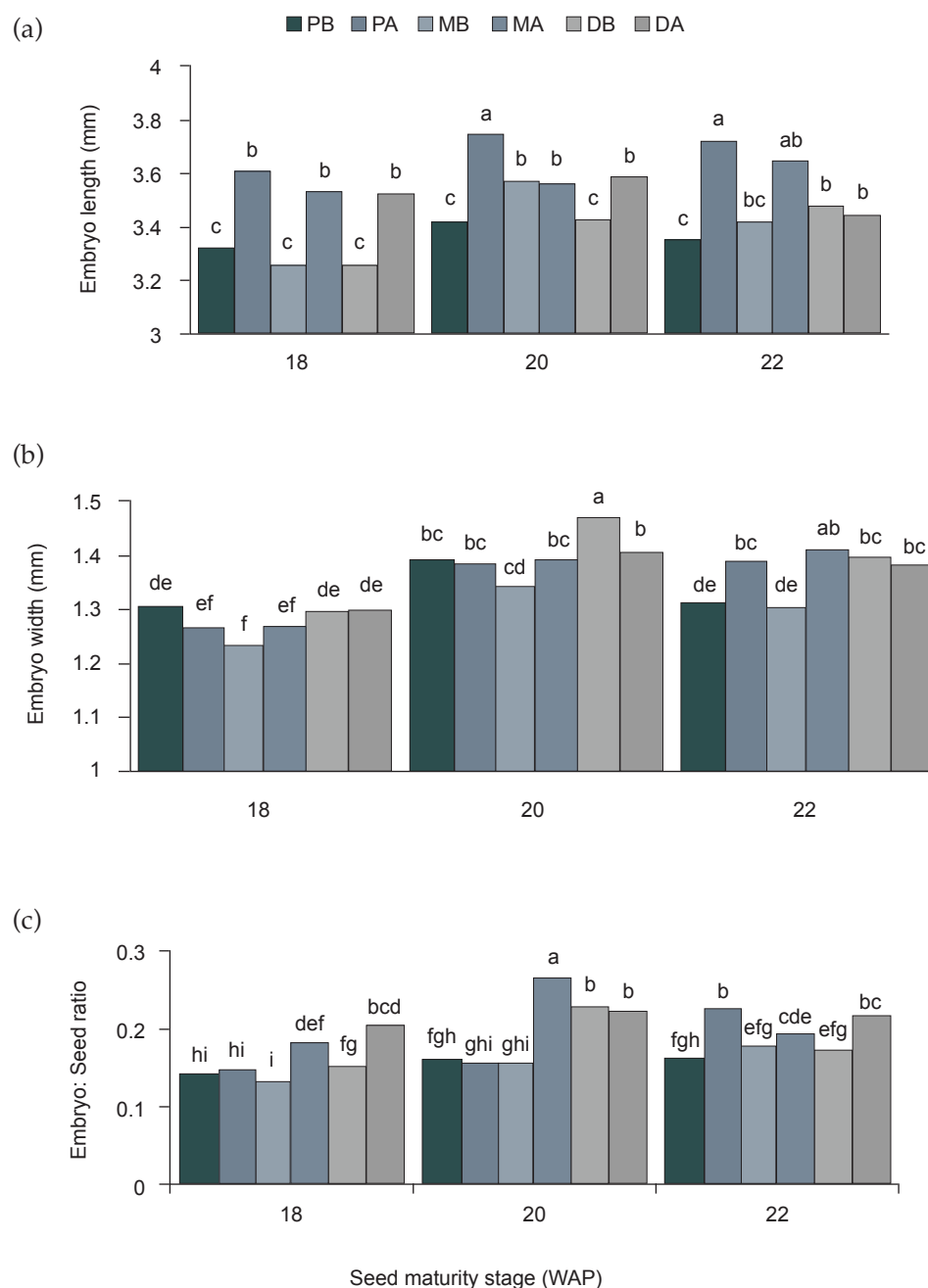


Figure 4. Diagram illustrating the seed developmental differences on seed endocarp colour and characteristics, based on its position in the spikelet and the bunch position in between two fronds. Arrow indicates the direction of increasing spatial restriction, thus resulting in smaller, rounded and less pigmented seeds at the base.

Seed producers generally harvest D×P bunches between 18 to 20 WAP, to ensure that fruitlets are still attached to the bunch and prevent loss from natural abscission (SDSAS 2020). Based on the seed endocarp colour assessment, it was evident that the white-coloured seeds gradually decreased with the maturity stages. This shows that natural pigmentation is still happening, even on the base section of the bunch. However, the process is slower due to the lower sunlight exposure as explained above. For this reason, the differences in seed endocarp colour could be due to natural developmental process and this only affected the external characteristics of the seed. Therefore, delaying the harvesting would greatly increase the percentage of black seeds, which translates to more saleable seeds since the white seeds are often perceived as being abnormal and mainly discarded during production (SDSAS, 2020). Thus, from the seed endocarp colour perspective, it would be advantageous for seed producers to harvest the seeds at 22 WAP. However, it is important to note that it also would significantly increase the incidence of loose fruits, which may cause potential losses of seeds during harvesting.

In this study, the evaluation for both morphological and germination parameters was compared at 18, 20 and 22 WAP; with a total of six weeks of evaluation. If we consider the whole oil palm seed developmental process from pollination to harvesting (approximately 20 weeks after pollination), the time of evaluation only

encompassed the final two weeks of the changes prior to and after the seed had reached the current standard harvesting stage. For this reason, most of the physical seed characteristics such as seed weight, seed length, seed width and operculum diameter were not significantly different, as the seed had already attained the maximum mass maturity at this point. It was reported that between 13 and 15 weeks after anthesis; the oil palm seed endosperm cavity had fully solidified, with the presence of an approximately 3 mm cylinder-shaped embryo underneath the operculum region (Kok *et al.*, 2013; 2015). In the case of sunflower seed, seed position was reported to influence the maturity as it happened progressively from the outer region towards the central part of the whorl (Gupta *et al.*, 2009). However, this is not the case for oil palm seeds. It was found that the embryo size increases with the seed maturity stage and is not influenced by the different seed positions. From the seed dormancy perspective, embryo development is often associated with both morphological and morpho-physiological dormancy attributes, having either rudimentary or underdeveloped embryos (Baskin and Baskin, 2014). Both embryo length and width showed up to 5% and 13% increment in size, from 18 to 20 WAP, respectively. If we look at the embryo-to-seed (E:S) length ratio, an average of 23% increment was recorded within two weeks of maturity, indicating an increase in embryo size in relation to the seed size (Figure 5).



Note: PB - proximal-base; PA - proximal-apex; MB - middle-base; MA - middle-apex; DB - distal-base; DA - distal-apex. Different letters indicate significant differences within each column, at a 5% probability level.

Figure 5. Interaction effect of seed maturity stages and seed positions on (a) mean embryo length, (b) mean embryo width and (c) mean Embryo: Seed (ES) Ratio.

An increasing E:S ratio is regarded as an indicator of morphological dormancy release, in seeds with underdeveloped embryos (Forbis *et al.*, 2002). For this reason, the germination was shown to be increasing, particularly from 18 to 20 WAP; with an average of 77.5% and 85.1%, respectively (Table 2). At 18 WAP, distal sections of the seeds had 5.0% higher germination as

compared to the proximal seeds, with 12% to 18% higher germination than the middle sections. Seeds that were harvested at 20 WAP showed a more uniform pattern, where no significant differences were observed at all seed sections (all germinated more than 83.0%) except for the proximal-base seeds; with 81.5% normal germination.

TABLE 2. PERCENTAGES OF SEED GERMINATION AND VIABLE EMBRYO

Seed stage	Seed position	Mean seed germination (%)	Mean viable embryo (%)
18	PB	74.00 ^{bc}	80.00 ^d
	PA	79.50 ^{ab}	87.50 ^{cd-e}
	MB	67.50 ^c	95.00 ^{a-c}
	MA	73.50 ^{bc}	100.00 ^a
	DB	85.50 ^a	95.00 ^{a-c}
	DA	85.00 ^a	97.50 ^{ab}
20	PB	81.50 ^{ab}	82.50 ^d
	PA	86.50 ^a	90.00 ^{b-d}
	MB	84.00 ^{ab}	97.50 ^{ab}
	MA	87.00 ^a	95.00 ^{abc}
	DB	88.50 ^a	100.00 ^a
22	DA	83.00 ^{ab}	100.00 ^a
	PB	88.50 ^a	95.00 ^{a-c}
	PA	85.00 ^{ab}	97.50 ^{ab}
	MB	87.00 ^a	95.00 ^{a-c}
	MA	81.50 ^{ab}	90.00 ^{b-d}
	DB	72.00 ^{bc}	85.00 ^{cd}
	DA	71.50 ^{bc}	95.00 ^{a-c}

Note: PB - proximal-base; PA - proximal-apex; MB - middle-base; MA - middle-apex; DB - distal-base; DA - distal-apex. Different letters indicate significant differences within each column, at a 5% probability level.

In contrast, 22 WAP seeds that were located at the distal sections of the bunch, had recorded significantly lower germination (less than 72%), in comparison to the proximal and middle sections. The percentage of abnormal and diseased seeds was less than 5% throughout all seed maturity stages and seed positions. In addition, the TZ test conducted in this study clearly showed that the differences between the estimated germination potential with actual germination had decreased from 18 WAP to 22 WAP, suggesting a reduction in the depth of primary dormancy in the seed (Baskin and Baskin, 2014; Flores *et al.*, 2011; Soares *et al.*, 2016; Vankus, 1997). At 18 and 20 WAP, seeds located at the middle and distal sections of the bunch (for both the apex and base section) showed more than 95% viable embryos. In contrast, the proximal-base and proximal-apex regions had recorded less than 90% embryo viability. However, at 22 WAP, no differences in embryo viability were recorded as all the seed positions had resulted in higher than 90% embryo viability, except for the distal-base section. This scenario showed that earlier harvesting even for only two weeks, could significantly increase the percentage of dormant seeds within the bunch.

The germination was not significantly different for seeds located at different positions, despite the clear morphological differences observed, particularly between the base and apex sections of the spikelets. However, the difference in seed morphology was not reflected in the germination parameters. Referring to *Table 2*, while the germination was different among some of the seed positions at 18 WAP (for example the distal sections had 5% higher germination as compared to the proximal seeds and 12% to 18% higher than the middle sections), however as the seeds undergo further maturation into 20 WAP, germination was found to be more uniform across different positions. Indeed, variation was observed for germination percentage and mean germination time; however, there was no direct correlation between the morphological characteristics with germination. It is possible that at 20 WAP, the seeds exhibit similar physiological composition across different sections of the bunch. For instance, the oil palm has identical endogenous hormones or enzyme concentrations that are required for germination, such as GA, ABA and α -amylase (Baskin and Baskin, 2014). The intrapopulation variation observed in this study

can also be explained from a seed evolutionary point of view. As we know, the seed is formed as a means of reproduction and survival for all angiosperms. There is a concept in the evolutionary study referred to as 'bet hedging', where the plant increases its chance of survivability in an uncertain environment by having a range of phenotypic characteristics (Slatkin, 1974). From the ecological context, this strategy is indeed advantageous as it serves as an adaptive mechanism towards any changing environmental conditions by having staggered seed germination, or seedling establishment in a population (Gremer and Venable, 2014; Simons, 2009). However, in agricultural production, this strategy is termed disadvantageous as it reduces uniformity, which ultimately results in suboptimal crop establishment and makes crop management more challenging (Finch-Savage and Bassel, 2016).

CONCLUSION

Harvesting at 20 WAP resulted in the highest germination percentage, with more uniform germination across different sections within the bunch. In addition, there was also an increase in the percentage of black-coloured seeds, in comparison to semi-white and white seeds, rendering harvesting at 20 WAP should increase the number of saleable seeds for the producers. Seed positions had shown significant effects on morphological characteristics such as seed weight, length and width. The base region of the spikelets exhibits smaller, rounded and more white-coloured seeds in comparison to the apex region, regardless of its position within the bunch. The structure of oil palm inflorescences was found to influence the morphological differences observed, due to the spatial variation during development. Despite the prominent variations within the bunch, these morphological differences did not correlate with the seeds' germination. Therefore, harvesting and processing the whole bunch at 20 WAP is recommended to the seed producers, based on the germination and seed endocarp colour evaluation.

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SOLKETAL LEVULINATE ESTER AS A POTENTIAL FUEL ADDITIVE FOR PALM BIODIESEL

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ABSTRACT

The use of Solketal levulinate ester (SoLE) as a potential cold flow improver (CFI) for palm biodiesel is studied. SoLE was added to palm biodiesel and several crucial palm biodiesel characteristics such as density, kinematic viscosity, pour point (PP), cloud point (CP) and cold flow plugging point (CFPP) were evaluated. SoLE was mixed with palm biodiesel at levels of 0.5%, 1.0%, 2.5%, 5.0%, 10.0%, 15.0% and 20.0%, except for CFPP, only at an additional level of 1.0%, 10.0% and 15.0%. From the study, it was observed that the best percent addition of SoLE depends on the respective parameter to be improved. The density of palm biodiesel, when blended with 0.5% to 2.5% SoLE has been shown to decrease the density of neat palm biodiesel and the density value is conformed to the EN ISO 12185 standard. Kinematic viscosity values were also enhanced with the addition of SoLE below 5.0% and the value was also within the standard range of kinematic viscosity of palm biodiesel, which must be in the range of between 3.5-5.0 mm² s⁻¹. The blending of palm biodiesel with SoLE at 15.0%-20.0% showed some promising effects by improving the pour point of palm biodiesel, between 2°C and 5°C, with a recommended 15.0% addition that is most economic. Similarly, the cloud point of palm biodiesel blended with SoLE at the same percentage addition showed a reduction between 3°C to 6°C. Palm biodiesel blended with SoLE at 15.0% addition showed a positive and noteworthy effect, especially on the improvement of the palm-biodiesel cold flow characteristics.

Keywords: biodiesel by-product, fuel additives, glycerol derivatives, palm biodiesel, solketal levulinate ester.

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INTRODUCTION

The constant upsurge in the human population has resulted in corresponding increased energy requirements, which led to extensive use of fossil fuel resources (Verma *et al.*, 2016). As a countermeasure, there has been a significant effort in expanding biodiesel as an option to conventional petroleum-derived diesel due to its environmental conformation. Biodiesel is made from renewable resources, for example, plant-based oils or animal

lipids. The world's largest palm oil producers are Indonesia and Malaysia. Hence, the most common feedstock of biodiesel in Malaysia is palm oil (Monirul *et al.*, 2015). Palm biodiesel like many other vegetable-based biodiesels, has many advantages over fossil fuels, such as being very less toxic, biodegradable, renewable and eco-friendly (Unlu *et al.*, 2018).

However, palm biodiesel also has some performance drawbacks due to the considerable amounts of saturated fatty acid content in its chemical composition (Jadhav *et al.*, 2022). High content of saturated fatty acids will give unsatisfactory cold flow characteristics to palm biodiesel, by having extreme cloud point (CP) and pour point (PP) (Tinprabath *et al.*, 2015). These drawbacks lead to the poor operability of palm biodiesel in cold weather. Therefore, to address these issues, many researchers have reported on the enhancement of palm biodiesel cold flow behaviour via blending with petrodiesel

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(Hazrat *et al.*, 2020), transesterification reaction of biodiesel, with branched or long-chain alcohols (Edith *et al.*, 2012), winterisation (Min *et al.*, 2019) and addition of pour point depressant additives (Nie and Cao, 2020).

As opposed to other methods, adding a cold flow improver is possibly the utmost appropriate and practical method to improve the cold flow property of biodiesel, because it is more economical and convenient. Cold flow improvers (CFIs), are additives that have a low melting point, branched and non-aromatic. They can reduce the influence of wax crystals on the fuel by modifying their shape, size, growth rate and agglomeration, thus inhibiting the formation of large crystals at low temperatures. Most of the CFIs promote the formation of small (10-100 μ m) needle-shaped crystals.

Cold flow properties of diesel fuel are generally determined by the following parameters *viz.* CP (cloud point), PP (pour point), and CFPP (cold filter plugging point) CP indicate the susceptibility of the fuel to plug the filters of the engine in a cold operating climate. On the other hand, PP refers to the bottom-most temperature at which the fuel becomes semi-solid and loses its flow characteristics. CFPP is the lowest temperature, expressed in degrees Celsius ($^{\circ}$ C), at which a given volume of diesel type of fuel still passes through a standardised filtration device in a specified time, when cooled under certain conditions (Verma *et al.*, 2016).

Recently, there are studies that reported the use of glycerol-based oxygenates as CFI for biodiesel, with the benefit of these compounds being renewable products derived from biomass (Muniz *et al.*, 2019). Converting glycerol to CFI also can be an alternative way to valorise glycerol to support the sustainability of the biodiesel industry. Converting glycerol into fuel additives can be made via the esterification reaction of glycerol with an organic acid such as acetic acid, levulinic acid, or the etherification and ketalisation reaction of glycerol along with alcohols or ketones respectively. This will generate a range of oxygenated glycerol compounds such as alkyl and acetyl ethers of glycerol and solketals (Saengarun *et al.*, 2018; Unlu *et al.*, 2017). These potential glycerol derivatives oxygenated additives have been evaluated as biodiesel cold flow improvers. Solketal (4-hydroxymethyl-2,2-dimethyl-1,3-dioxolane) respectively has attracted more attention recently, due to its unique features in improving cold flow properties and flash point temperatures of both diesel and biodiesel as well as its blends (Aghbashlo *et al.*, 2018). It was also disclosed in a study that octane number and gum growth in gasoline decreased when oxygenated fuel additive from glycerol namely solketal was added. (Ilgen *et al.*, 2017; Saengarun *et al.*, 2017). Diesel fuel when blended with glycerol-based additives was

reported to minimise particulate emissions, improve lubricity and engine performance (Çakmak and Özcan, 2020; Neamtu *et al.*, 2019)

Glycerol-based oxygenated compound that will be discussed in this article is classified as a solketal ester. Generally, solketal ester is made from solketal and organic acid or ester. Solketal is a derivative of glycerol, which is prepared from a reaction between glycerol and acetone. Whereas compounds with carbonyl groups such as fatty acids, ketones, and anhydrides that have been studied to produce solketal esters are caprylic, lauric, palmitic and stearic acids, acetic anhydride, butyric anhydride, butanone, cyclopentanone, 4-methyl-2-pentanone and 3,3-dimethyl-2-butanone (Perosa *et al.*, 2016). The selection of the carbonyl group from each study is arbitrary.

On the other hand, to the best of our knowledge, no study was published that described solketal levulinate ester as a cold flow improver additive, particularly in palm biodiesel. Solketal levulinate ester (SoLE) is a 100% bio-based product prepared from a reaction between solketal and methyl levulinate. Methyl levulinate is derived from biomass resources such as palm biomass including leaves, fronds and trunks (Liang *et al.*, 2020). Therefore, in this work, we present the study of SoLE as a CFI additive to enhance the palm biodiesel cold flow characteristics. Effects on density value, kinematic viscosity, acid value, cloud point, pour point and cold filter plugging point were assessed.

MATERIALS AND METHODS

Solketal (99.8% purity), methyl levulinate, and sodium carbonate have been acquired from Sigma Aldrich, USA. Diethyl ether (99.0%) from ChemAR, Kuala Lumpur, Malaysia. Palm biodiesel was sourced from a local plant. All chemicals were directly used.

Synthesis Ester of Solketal Levulinate (SoLE)

SoLE was prepared from a method reported by Mariam *et al.* (2021). In brief, the reaction was conducted in a 50 mL one-necked round bottom flask assisted with a magnetic stirrer. Solketal, methyl levulinate and sodium carbonate were put in and heated to the preset temperature of 140 $^{\circ}$ C in the said flask and were heated for 4 hr. Reagents were weighed up to the preferred molar ratio. The molar ratio of methyl levulinate to solketal used in this study was 1:3 where the percentage of catalyst added was 1.5% of solketal weight. An oil bath assisted by thermometer was used in monitoring the temperature of the reaction. A low vacuum was applied at the end of the condenser to capture and observe the released methanol from the

reaction. The reaction was observed using thin-layer chromatography. As the reaction completed, the product obtained was washed with distilled water to wash away sodium carbonate. This was followed by a solvent extraction step to extract SoLE using diethyl ether as solvent. These steps were repeated three times. After washing, the non-aqueous layer was collected and purified using a rotary evaporator. The product was analysed with gas chromatography (GC), ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and Fourier Transform Infrared (FTIR) spectroscopy. The yield of SoLE was determined to be about 75% and the purity of prepared SoLE was about 95%.

Analysis and Characterisation of SoLE

FTIR. Determination of the SoLE functional groups was conducted via Fourier Transform Infra-Red analysis using Spectrum 100 FT-IR Spectrometer Perkin Elmer and the spectra were collected using 64 scans at 4 cm^{-1} resolution in the range of $650\text{--}4000\text{ cm}^{-1}$.

GC analysis. GC analysis was conducted to confirm the quantitative percentage of SoLE. The GC (Agilent System 6890N Network GC System) was set up with a ZB-5HT INFERNO ($30\text{ m} \times 250.0\text{ }\mu\text{m} \times 0.2\text{ }\mu\text{m}$) capillary column and detector of flame ionisation. The temperature was programmed at oven temperature, 80°C ; initial temperature, 80°C ; the heating rate at $10^\circ\text{C min}^{-1}$; final temperature 315°C ; injector temperature, 300°C ; detector temperature, 325°C ; carrier gas, helium at 40.0 mL min^{-1} . For sample preparation, it was diluted with dimethylformamide and dichloromethane with a ratio of one to one. The percent composition of the sample was confirmed conferring to GC chromatogram percent area.

^1H and ^{13}C NMR analysis. Proton (^1H) and carbon (^{13}C) NMR spectroscopy were recorded on JOEL JNM-ECZ600R at 600 MHz and 150 MHz, respectively at 298 K with approximately 10% w/v solutions in deuterated chloroform (Sigma, USA) NMR solvents. Chemical shifts are quoted in ppm relative to internal standard tetramethylsilane (TMS) (Sigma, USA) and reference to the residual solvent. All coupling constants are quoted in hertz (Hz). The ^1H and ^{13}C NMR assignments were routinely confirmed by $^1\text{H}\text{--}^1\text{H}$ (COSY) and $^1\text{H}\text{--}^{13}\text{C}$ (HMQC) data.

Chemical Analysis

Acid value. Acid value (AV) is defined as the number in milligrams of potassium hydroxide (KOH) (mg) (System, Malaysia) needed to neutralise the free fatty acid present in the unit mass (g) of a chemical

substance. MPOB Test Method c2.7: 2004 was used as the test method.

The acid value is conveyed as Equation (1):

$$\text{Acid value} = \left(\frac{56.1 \times V (\text{mL}) \times M}{m} \right) \text{mg KOH g}^{-1} \quad (1)$$

where V is the KOH volume, M is concentration of KOH solution, m is the mass of the sample. AV analysis was made in duplicate and presented as mean value.

Physicochemical Analysis

Density. Density was testified using DMA 5000 M (Anton Paar, UK) density meter. By using a syringe, 2 mL of product was transferred to a measuring tube until a drop emerged from the sample outlet adapter. Any presence of bubbles was circumvented. The measurement was conducted in duplicate for every product at 25°C and finalised as a mean value.

Kinematic Viscosity

Measurement of kinematic viscosity (KV) was according to ASTM method D 445–12. PMT Tamson NVB Classic (Normalab, France) was used to conduct the test. The viscometer bath was set between 40°C and 100°C (Constant temperature bath, Cannon Instruments, State College, PA). The test was duplicated for every product and the final reading was recorded in average value. ASTM method D446 was used as a standard to select types of viscometry capillary (Ubbelohde capillary viscometer, Cannon Instruments, USA).

Cloud Point (CP)

Cloud point (CP) of SoLE was tested via the PP Classic purchased from Norma lab (Belgium) following the official ASTM method D 2500–09. The product (50 mL) was put in a test container and examined at 1°C decrement.

Pour Point (PP)

Pour point (PP) was evaluated in reference to ASTM method D 97–12 using PP Classic (Normalab, Belgium) with an efficiency of 3°C . The analysis was conducted by putting a test container containing 50 mL of sample into a cylinder immersed in ethanol (95%) as the cooling media. The analysis container was clutched horizontally for 5 s in every 3°C reductions and ceased when samples were no longer flowing. The temperature where the product is not flowing would be added to 3°C and denoted as the PP.

Cold Filter Plugging Point

Cold filter plugging point were determined based on EN 116. The CFPP is the highest temperature at which a given volume of fuel fails to pass through a standard filtration device in a specific time, when cooled under standard conditions.

RESULTS AND DISCUSSION

Synthesis of levulinate ester via an esterification reaction between levulinic acid and short chain alcohol has been previously reported using different homogeneous acid catalysts, for example, sulphuric acid (H_2SO_4), hydrochloric acid (HCl) and phosphoric acid (H_3PO_4) (Pileidis and Titirici, 2016) and various heterogeneous acid catalysts, such as sulphonic acid functionalised materials (Song *et al.*, 2015). However, we observed that the esterification of solketal with levulinic acid in the existence of an acid catalyst gave sub-standard yields of SoLE. This might be due to hydrolysis of solketal that might occur under an acidic state developing unwanted by-products. This corresponds to the result described by Noor Armylisas *et al.* (2021) where utilisation of acid catalyst will accommodate more side reactions, for example, fatty acid degradation (oxidation, dimerisation) or from polymerisation or dehydration of glycerol that turns into a higher polymer of glycerol or acrolein. Therefore, in this study, SoLE was synthesised using a base-catalysed transesterification reaction as we previously established in our lab. The properties of SoLE with 95% purity were reported previously (Mariam *et al.*, 2021). Figure 1 showed the chemical reaction equation for transesterification of solketal with methyl levulinate to obtain solketal levulinate ester.

Fuel Properties of Biodiesel Blended with SoLE as Cold Flow Improver Additive

The properties of the palm biodiesel with the addition of v/v SoLE were investigated as a CFI additive. Addition of SoLE was at 0.5%, 1.0%,

2.5%, 5.0%, 10.0%, 15.0% and 20.0% respectively. The fuel properties, for example, density (25°C) (EN ISO 12185), viscosity (40°C) (EN ISO 3104), acid value (EN 14104), pour point (ISO 3016), and cloud point (ASTM D2386) were measured and concluded in agreement with the established methods. The biodiesel parameters were established according to EN 14214 and ASTM D6751 standards. Fuel property tests and measurements were repeated two times and they were reported as a mean value.

Density

Density is a crucial fuel characteristic for biodiesel. Biodiesel has been deliberated as renewable fuel with both benefits and disadvantages. However, the disadvantage of biodiesel such as high density at low temperature affects diesel engines and fuel systems destructively. Thus, the reduction of the density of biodiesel fuel in unmodified diesel engines is crucial (Pham *et al.*, 2018). Consistent with the standard implemented (EN ISO 12185), the density value of biodiesel must be in the range of between 860.0-900.0 kg m^{-3} . The density of SoLE and neat palm biodiesel use in this study are 1097.6 kg m^{-3} and 877.0 kg m^{-3} respectively. The density of palm biodiesel with the incorporation of different amounts of SoLE was between 870.0-908.0 kg m^{-3} except for biodiesel with the addition of 20% SoLE (Figure 2). However best performance was observed with addition of SoLE at 0.5%, that gave density value of 869.5 kg m^{-3} . The addition of SoLE has reduced the density of neat palm biodiesel by 7.0 kg m^{-3} . As described by Alptekin and Canakci (2008), density of the material (oil) should be very low (860.0-900.0 kg m^{-3}) to avoid engine failure. High density can affect the process of atomisation during biodiesel combustion, resulting in lower engine performance and increased NOx emissions.

Properties of palm biodiesel when added with SoLE has showed to comply with the international standards, even with high density of SoLE (1096.6 kg m^{-3}), proving its compatibility to be used in a diesel engine.

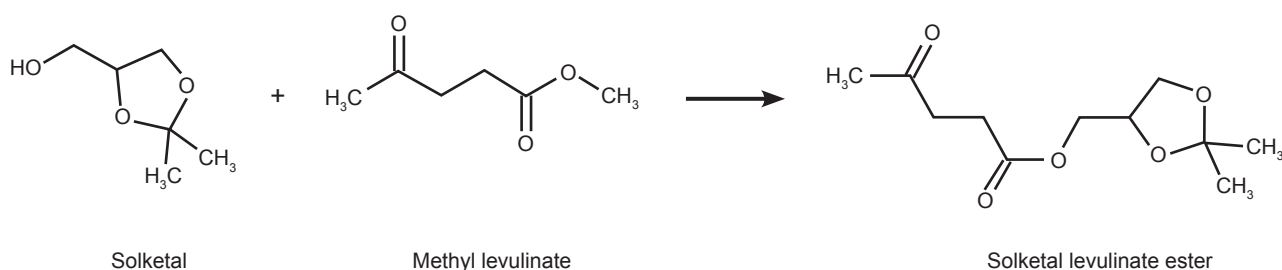


Figure 1. Reaction scheme of transesterification of solketal with methyl levulinate to obtain solketal levulinate ester.

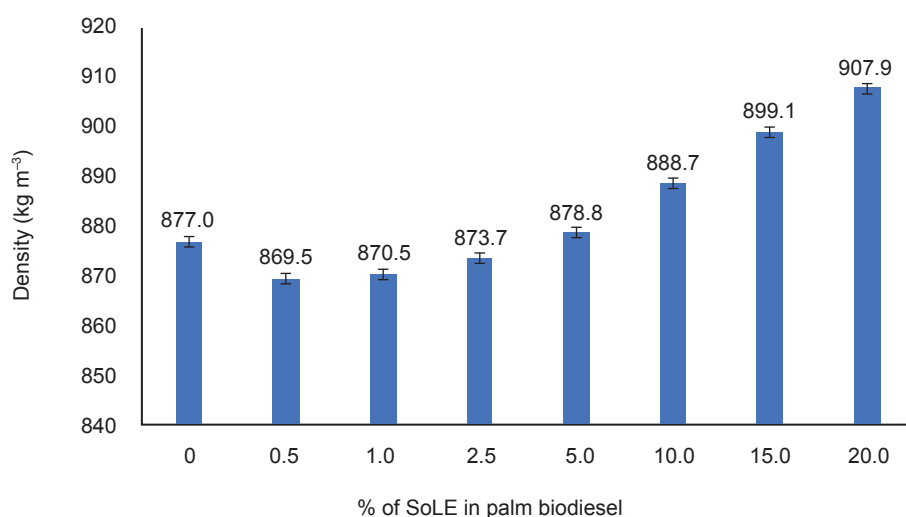


Figure 2. Effect of SoLE amount on the density of palm biodiesel.

Therefore, the results showed that palm biodiesel blended with 0.5% to 2.5% of SoLE have improved the density of palm biodiesel to the minimum limit of the standard specification value. Addition of SoLE has showed to improve the characteristic that contribute to the engine performance. This indicated the noteworthy potential of using SoLE as an effective CFI additive for palm biodiesel.

Study by Garcia *et al.* (2008) has reported that addition of pure solketal at 5% to neat biodiesel has showed to increase the density of the neat biodiesel. They also extent their study on the addition of solketal acetate and triacetin mixture at 2/3 ratio and similar result was observed where the density of the neat biodiesel increased. In terms of density characteristics, they concluded that the addition of acetyl group to solketal does not help in improving the biodiesel density. In contrast with our study, the addition of levulinate group to solketal had improved the density of neat biodiesel. Nevertheless, our finding is coherent with the study by Ali *et al.* (2014), that reported the density decreased with the addition of oxygenates additive such as alcohol and ether to palm oil methyl ester. Venkatesan *et al.* (2017) in their study also revealed that, the density of biodiesel blends from *Jatropha* oil were gradually reduced with the addition of oxygenates. Generally, addition of suitable oxygenates additives, such as glycerol based oxygenates compound to palm biodiesel, can potentially reduce its density. The basic factors that affect the liquid density are atomic weight of the element or the molecular weight of the compound and the distance between atoms (Inter-atomic distances) or molecules (Inter-molecular spaces). Therefore, the differences of the effect given by respective oxygenates additives are reported might be due to the basic factors as stated above.

Kinematic Viscosity

Kinematic viscosity is the crucial key feature of biodiesel which measures the resistance of the biodiesel to flow (Kassem and Çamur, 2016). High kinematic viscosity of fuel has a detrimental effect in low-temperature weather, where viscosity escalates when the temperature is low (Liu, 2015). According to the kinematic viscosity standard for biodiesel, the kinematic viscosity values must be in the range of 3.50 and 5.00 mm² s⁻¹. The kinematic viscosity of SoLE and palm biodiesel used in this study were 7.32 mm² s⁻¹ and 4.64 mm² s⁻¹ respectively. Figure 3 showed the deviations in the kinematic viscosity value of palm biodiesel *versus* the SoLE amount. It was observed that the addition of SoLE at all percentages tested (0.5% to 20.0% v/v) resulted in kinematic viscosity of between 4.51-4.73 mm² s⁻¹. These values are within the standard specification for kinematic viscosity of biodiesel. However, it was also discovered that at lower percentage addition of SoLE (0.5% to 5.0%), the viscosity of palm biodiesel had slightly enhanced.

Palm biodiesel blended with SoLE has showed to slightly improve the viscosity values that is also within the standard limits of the kinematic viscosity standards for biodiesel. This result demonstrates that, the process of improving cold flow property by SoLE addition would not give an adverse effect on other fuel properties such as kinematic viscosity and density.

Our findings supported the previous study from Garcia *et al.* (2008) where they have reported that introduction of an acetyl group in the free OH of solketal has been an effective solution to improve the viscosity of palm biodiesel. The viscosity also complies with ASTM D6751 and EN

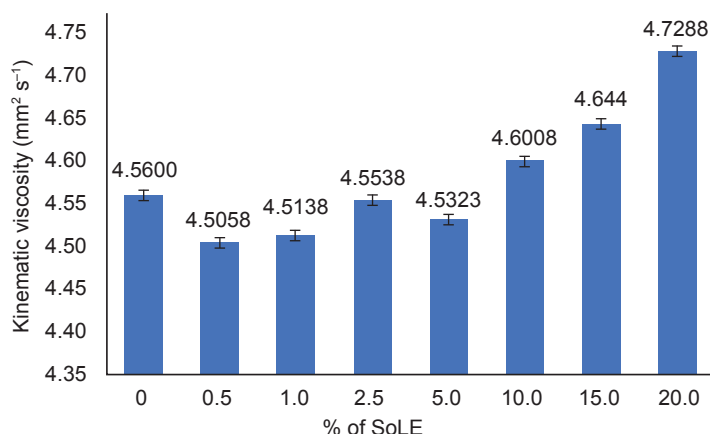


Figure 3. Effect of SoLE amount on the kinematic viscosity of palm biodiesel.

14214 Standards. To the best of our knowledge, no previous reports had discussed on how the addition of CFI additive had affected the kinematic viscosity of palm biodiesel. This might be due to the raw materials having the greatest influence on the biodiesel viscosity. Fatty acid composition determines the degree of saturation while the composition determines the degree of saturation. Viscosity increases with increasing degree of saturation (Ayeter *et al.*, 2015).

Acid Value

The acid value (AV) measurements were conducted following EN 14104 analysis method. The maximum AV of palm biodiesel, as stated in the above analysis method is 0.5 mg KOH g⁻¹. Typically for biodiesel, the acid number derives almost exclusively from the fatty acid content due to the hydrolysis (depend on the amount of water that may likely be present in the biodiesel) of ester linkages in both the triglyceride feedstock and the biodiesel during its manufacturing (Mahajan *et al.*,

2006). Figure 4 demonstrates the variations of AV with the addition of various amounts of SoLE in the fuel mixture. The addition of 0.5% to 20.0% of SoLE into palm biodiesel resulted in acid values of blends to be approximately 0.6 mg KOH g⁻¹. The result shows a marginal difference in the acid number of biodiesel blends in comparison with the maximum limit allowed in EN 14104 standard. This might be due to the contribution of AV from levulinic acid which may originate from the hydrolysis of SoLE that produce solketal. Palm biodiesel blended with SoLE only showed a marginal deviation from the standard specification for the AV. This can be improved by maximum stripping off any compound that may contribute to the higher AV of palm biodiesel.

Pour Point

The pour points of biodiesel were tested according to the ISO 3016:2019(E) analysis method. The pour point (PP) of a fuel is defined as the lowest temperature at which it becomes semi-solid and

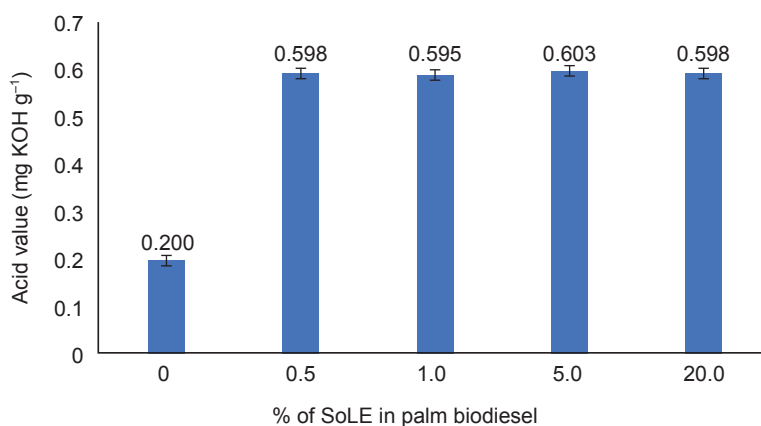


Figure 4. Effect of SoLE amount on the acid value of palm biodiesel.

loses its flow characteristics. When the temperature is further reduced, the crystal nuclei aggrandise and inhibit the free-flowing of fuel (Verma *et al.*, 2016). From our lab analysis, palm biodiesel PP was 19°C and this value was similar as reported by Verma *et al.* (2016), which was 19.7°C. Figure 5 shows the pour points of palm biodiesel blended with SoLE at levels of 0.5% to 20.0%. The results showed reductions of PP values between 2°C to 5°C, in comparison with the neat palm biodiesel. The lowest PP was observed when 15.0% and 20.0% of SoLE was added. The results observed in our study are in accordance with the observation conveyed by Cornejo *et al.* (2017) that evaluated different glycerol-based oxygenated compounds such as solketal, triacetin and mixtures of esters mono-, di- and tri-acetylated glycerol (MAG, DAG, and TAG) and a mixture of ethers such as mono-, di- and tri-*tert*-butyl glycerol (MTBG, DTBG, and TTBG). Their study conveyed that glycerol-based oxygenated compounds stated above (Figure 6) have been proven as PP and cloud point improvers, especially in biodiesel and their blends. Another study by Silva *et al.* (2010) indicated that the addition of butanal/glycerol acetal (one of the glycerol-based oxygenated compound family) had reduced the PP of animal fat biodiesel in 5°C.

It should be highlighted that as the palm biodiesel temperature was reduced, polar groups of oxygen-bearing functional groups, be it esters, alcohols, ketones, and ethers from the glycerol-based oxygenates class, would come in contact with the polar methyl ester groups to reduce and arrange the cluster size of the hydrocarbon crystals. Thus, pour and CP of biodiesel were reduced when the crystal size of the saturated fatty methyl ester was lessened (Saengarun *et al.*, 2017).

Glycerol-based oxygenated additive generally has been reported to improve the pour point of biodiesel. However, the advantage of SoLE over other glycerol-based oxygenated additive as raw material to produce SoLE both can be obtained from palm derivatives, which is glycerol from palm oil and levulinic acid from palm biomass respectively. This can be a sustainable approach to improve palm biodiesel industry directly and palm oil industry at whole.

Cloud Point (CP)

CP is the evaluation of the critically low temperature at which fuel develops the first particles of wax crystals and cloudiness is observed, as the temperature of the fuel gradually decreased under standard conditions (Saeedi Dehaghani and Rahimi, 2019). The best CP should be obtained in the range of -3 to -12 according to standard. Palm biodiesel contains high content of palmitic acid that contributes to the high CP of palm biodiesel at 21°C (Verma *et al.*, 2016). Figure 7 presents the CP values of palm biodiesel, blended with various amounts of SoLE. It was observed that, as the amount of SoLE elevated, the CP of palm biodiesel depreciated from 21°C to as low as 15°C. Lowest CP was observed when palm biodiesel was blended with 15% and 20% of SoLE. From this result, we reckon that, it is likely that SoLE had depresses the palm biodiesel CP. SoLE played a role in delaying the agglomeration of the wax crystals in palm biodiesel, or in other words, it alters the crystal shape and size which will inhibit lateral crystal growth. This effect is of utmost interest showed by SoLE. In this study, the CP measurements were performed according to the ASTM D 2386 analysis method. According to this standard, the CP

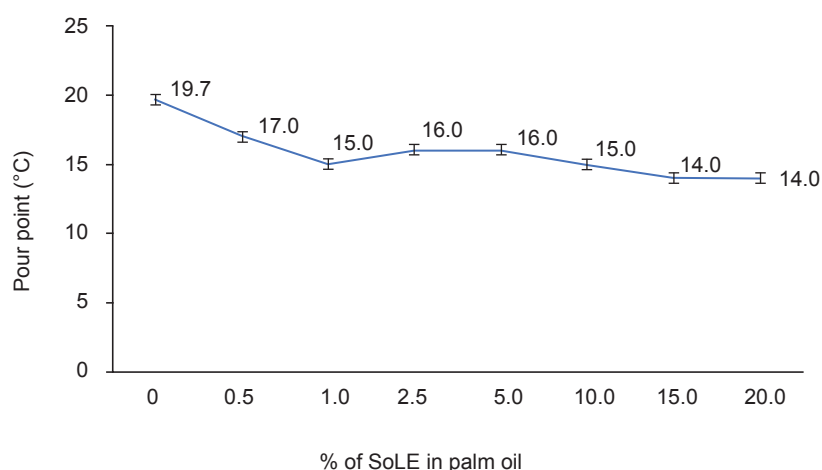


Figure 4. Effect of SoLE amount on the acid value of palm biodiesel.

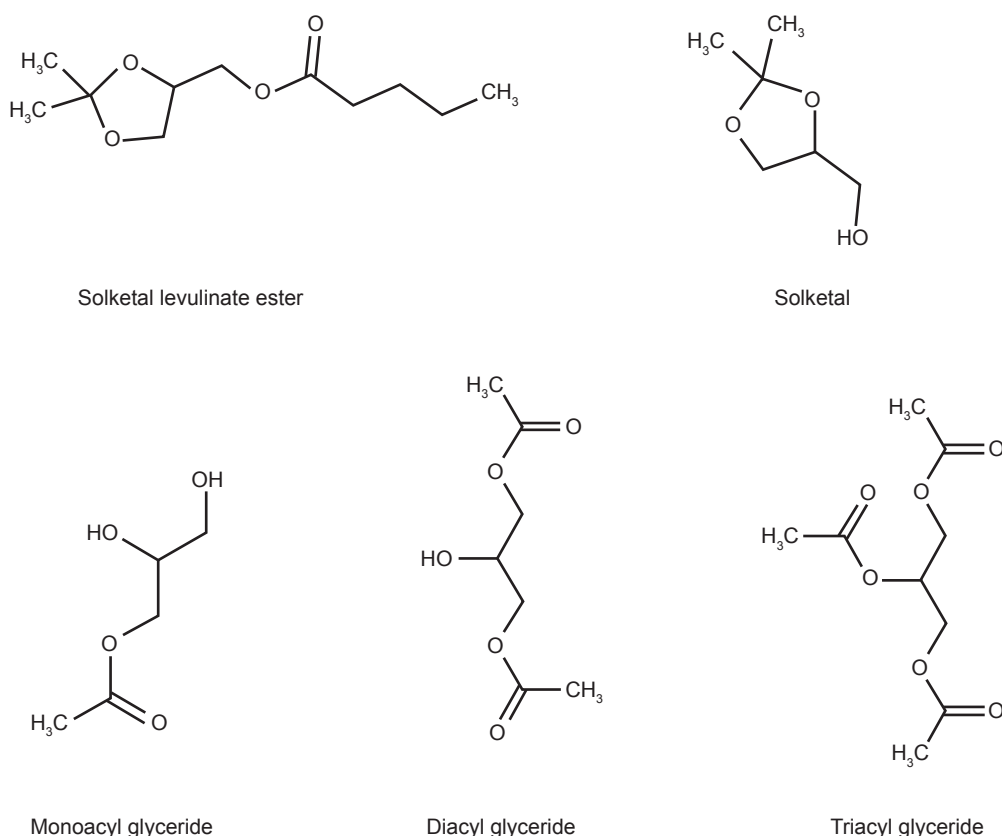


Figure 6. Chemical structure of glycerol-based oxygenated compounds.

value of biodiesel should be between 5°C and -65°C. From the results obtained, the addition of SoLE has improved palm biodiesel CP even though it has slightly diverged from standard. This result showed the potential of SoLE as a cold flow improver of palm biodiesel. This finding is in accordance with Klepáčová, *et al.* (2003) whereby their study stated that the glycerol alkyl ethers (glycerol oxygenates with ether functional group) had reduced the CP when added to biodiesel with 5°C reduction while SoLE at 6°C. From this study, to improve the CP of palm biodiesel, the optimum suggested addition of SoLE is 15%. As described earlier, the advantage of SoLE is the raw material from palm based origin, which can reduce the dependency on imported chemical additive to improve the CFI of palm biodiesel.

Cold Filter Plugging Point

Cold filter plugging (CFPP) point is described as the minimum temperature at which 20 mL of sample passes through the filter in 60 or less (Leng *et al.*, 2020). According to the standards CFPP value,

it should be +5°C or as low as -20°C when compared with the neat biodiesel CFPP value. Table 1 shows CFPP value of neat biodiesel and CFPP value of neat biodiesel when added with 1%, 10% and 15%. The result conveyed that no significant improvement of CFPP value were observed at any percentage of SoLE added. With these results, SoLE can be considered as not an effective additive to improve CFPP of neat biodiesel.

Besides that, Garcia *et al.* (2008) also reported that the mixture of solketal acetate and triacetin that they synthesised from solketal and acetic anhydride also showed no significant effect in improving CFPP of neat biodiesel.

Edith *et al.* (2012) reported that CFPP reducing additives, called “CFPP depressants”, act during nucleation by altering the structure of the crystals formed from an orthorhombic shape to a needle-like shape, hence no blocking to fuel filters. Thus, they inhibit the growth of the crystals formed, to ensure that they remain in a fine suspension rather than gelling up. They are usually used with wax anti settling agents. From this we can conclude that SoLE does not act as how CFPP depressant works.

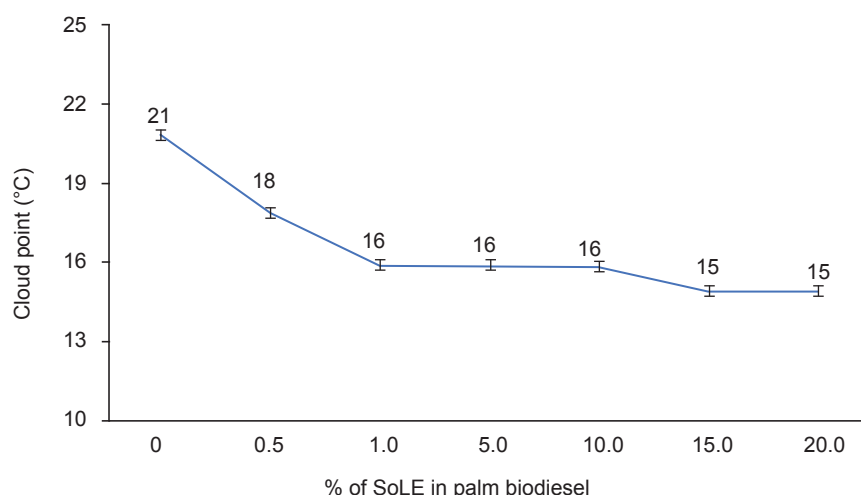


Figure 7. Effect of SoLE amount on the cloud point of palm biodiesel.

Although SoLE does not improve CFPP very well, SoLE works quite well as PP additive, and as CP additive. This might be because SoLE does not alter the shape of the crystals formed; instead, they collect on the surface of the crystals formed to hinder their growth and prevent gel network formation.

TABLE 1. EFFECT OF SOLE ADDITION ON COLD FILTER PLUGGING POINT OF NEAT PALM BIODIESEL

Sample	Cold filter plugging point (°C)		
	Test 1	Test 2	Test 3
Biodiesel 100%	11	11	11
SoLE 1%	10	10	10
SoLE 10%	9	9	9
SoLE 15%	9	10	9

CONCLUSION

This study has shown that SoLE can be added to palm biodiesel as CFI additive, with best percent addition suggested at 0.5% to 5.0% for improvement of density and kinetic viscosity, and 15.0% for improvement of pour point and cloud point. Addition of SoLE does not give any prominent effect on CFPP value. Density of palm biodiesel when blended with 0.5% SoLE gave density value of 869.5 kg m^{-3} and the value showed to conform to the EN ISO 12185 standard with the reading obtained at 899.1 kg m^{-3} . Kinematic viscosity had slightly enhanced at lower percentage addition of SoLE (0.5% to 5.0%), and the values also conformed with the standard which must be in the range of 3.5 to 5.0 $\text{mm}^2 \text{ s}^{-1}$. The blending of palm biodiesel with SoLE at 15%-20% percentages also showed promising effects by improving the pour point of palm biodiesel between 2°C and 5°C, and

15% is considered to be the most economic and recommended. Similarly, the cloud point of palm biodiesel blended with SoLE at same percentage addition also showed a reduction of between 3°C to 6°C.

SoLE can improve the low temperature properties of biodiesel, which will probably promote biodiesel utilisation in cold weather. In addition, SoLE was able to improve the cloud point and pour point of palm biodiesel. Density and kinematic viscosity conform to the standard specifications measured in this study, nonetheless, acid value showed marginal deviation value from the specification. In summary, SoLE could be regarded as a potential bio-based cold flow properties improver to improve the cold flow properties of palm biodiesel.

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CONSOLIDATION OF DEEP EUTECTIC SOLVENTS WITH MICROWAVE FOR THE EXTRACTION OF FERULIC ACID FROM THE FRUITLETS OF OIL PALM

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ABSTRACT

This article reports the extraction of ferulic acid from oil palm fruitlets by using deep eutectic solvent (DES) with microwave assistance. The difference in the amount of ferulic acid extracted from unsterilised and sterilised oil palm fruitlets using DES with microwave assistance was not significant, signifying that sterilisation during palm oil milling did not have much effect on the content of ferulic acid in the palm fruitlets. Acetic acid (AA) is a better hydrogen bond donor than citric acid in choline chloride (ChCl) DES whereby higher amount of ferulic acid could be extracted from oil palm fruitlets when ChCl-AA was used as the extraction medium (ca. 40.3 mg/g versus ca. 34.4 mg/g). The optimum conditions for the extraction of ferulic acid from palm fruitlets were by using ChCl with AA containing 30 wt.% water, heated with microwave at 60°C for 6 min. Ferulic acid extracted from unsterilised oil palm fruitlets exhibited higher radical scavenging activity (92%-95%) than sterilised fruitlets extract (88%-92%), indicating that although the sterilisation process did not significantly affect the content of ferulic acid in the oil palm fruitlets, the bioactivity of the ferulic acid was slightly affected.

Keywords: deep eutectic solvent, extraction, ferulic acid, microwave, palm fruitlets.

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INTRODUCTION

Palm oil milling starts with sterilisation to halt the enzyme activities responsible for the rise in free fatty acids and to soften the fruits to facilitate detachments from the stalk. However, the high temperature (130°C to 160°C) could also affect the non-oil components in the palm fruitlets.

Ferulic acid, a flavonoid polyphenolic compound found in several parts of the oil palm, could be one of the non-oil components affected by the sterilisation process of the palm fruits (Mohd Aanifah, 2014; Neo *et al.*, 2010; Ng, 2020; Ng and Nu'man, 2021; 2022a). Ferulic acid is a valuable component in nutraceutical, pharmaceutical and cosmeceutical industries due to its anti-microbial, antioxidative properties and

could act against inflammation (Mancuso, 2007; Neo *et al.*, 2008, 2010; Ou and Kwok, 2004). Efforts have been made in the past to extract the ferulic acid from various parts of the oil palm including, fruitlets, fronds, empty fruit bunches and palm pressed mesocarp fibre by way of solvent extractions such as alkaline hydrolysis and methanolysis (Ng, 2020; Ng and Nu'man, 2022b; Stavova *et al.*, 2017; Sun *et al.*, 2001; Tang *et al.*, 2014; Zhou *et al.*, 2019).

As ferulic acid could exist in free form as well as attached to lignin and other biopolymers in plants, efforts for its extraction need to take into consideration the penetration and cleavages of these links by the extraction medium. As much as 3800-4200 mg/kg ferulic acid could be obtained from palm empty fruit bunch by way of hydrolysis with alkali while 250-514 mg/kg free ferulic acid was obtained by way of methanolic extraction (Mohd Aanifah, 2014; Ng, 2020). Ng and Nu'man (2022b) also reported the extraction of ferulic acid from palm pressed fibre by way of alkaline extraction. Although

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extraction using alcohol is relatively simpler, only ferulic acid in free form could be extracted with this method. Alkaline hydrolysis, on the other hand, involved harsh conditions and ferulic acid that was extracted using alkali cannot be termed as 'natural'. As such, there is a need for a mild technique such as using deep eutectic solvent (DES) to extract the ferulic acid from the oil palm fruits.

DES, a eutectic mixture system of Lewis or Brønsted acids and bases consisting of a variety of anionic and/or cationic species, is capable of cleaving the link of lignin and/or biopolymers while protecting the bioactives from plant materials (Cvjetko *et al.*, 2015; Paiva *et al.*, 2014). Other advantages of DESs include being made up of natural, cost effective, safe and simple compounds. These enabled the DESs to be excellent solvents for the extraction of bioactives from plants, especially phenolic compounds which dissolved better in DESs compared to water or lipids (Smith *et al.*, 2014; Young *et al.*, 2011). DESs could dissolve a high variety of compounds as they can both donate and accept protons and electrons (Bi *et al.*, 2013; Dai *et al.*, 2013a; 2013b; Woo *et al.*, 2015). Another advantage of using DESs is that it offers excellent stability to the phenolic compounds extracted rendering it to be a possible novel formulation for pharmaceutical and food products (Liu *et al.*, 2019).

Besides conventional heating such as reflux, DES could complement microwave heating for the extraction of phytochemicals from plant samples. The potential for microwave heating with DES for extraction was supported by the study on the extraction of hydrophilic from *Radix Salviae miltiorrhizae* whereby it was reported that the yield obtained using DES with microwave heating was higher and at a shorter duration (11 min) compared to conventional extraction techniques (Chen *et al.*, 2016). Sharma and Dash (2021) also reported the completion of the extraction of certain phytochemicals from black jamun pulp in 4 min with DES under microwave assistance. This was due to the faster release of intracellular compounds as a result of plant cell rupture brought about by microwave heating and DES penetration. A study by Xie *et al.* (2019) also confirmed such phenomena whereby similar yield (~2.3 mg/g) of ferulic acid from chuanxiong could be obtained in just 20 min with microwave assisted DES extraction compared to non-microwave assisted heating which required 4 hr to be completed. Ng and Nu'man (2021; 2022a) reported a method for the extraction of ferulic acid from palm pressed fibre using microwave assistance whereby the extraction was completed in just 9 to 15 min compared to 6 hr by reflux (Ng and Nu'man, 2021; 2022a). This showed that the use of microwave heating could significantly increase extraction efficiency by drastically reducing extraction duration. Therefore, microwave

assisted DES could be further developed as an effective and efficient method for the extraction of phytochemicals.

Although the use of DESs in oil palm processing is getting more popular, there is yet any study on the extraction of ferulic acid from the mesocarp of oil palm fruitlets using DES with the aid of microwave heating. This paper aimed to study the impact of sterilisation on ferulic acid, as well as the factors, such as type of DES, heating temperature and duration, that could affect the extraction of ferulic acid from oil palm fruitlets using DES heated with microwave.

MATERIALS AND METHODS

Materials

Whole oil palm fruitlets (sterilised and unsterilised) were obtained from a palm oil mill in Malaysia. Choline chloride (ChCl), acetic acid (AA) and citric acid (CA) used in the study were of analytical or chromatography grades, obtained from Merck (Darmstadt, Germany).

Synthesis of DES

DES (choline chloride - acetic acid, ChCl-AA) was prepared following the method outlined by Abbot *et al.* (2004) with slight modifications. ChCl and AA in equal molar ratio were heated and stirred at 85°C until the mixture become homogenous and colourless. The liquid was then left to cool. Distilled water was then added into the ChCl-AA to obtain a range of DES containing 0 wt.% to up to 50 wt.% water. The same procedure was used to prepare ChCl with CA as a hydrogen bond donor (ChCl-CA).

Viscosity Analysis of DES

The viscosities of all the DESs prepared (ChCl-AA and ChCl-CA) were determined at 40°C with a Herzog Automated Multi Range Viscometer (HVM 472).

Sample Pre-treatment

Fresh whole oil palm fruitlets were peeled to obtain their flesh (mesocarp) while the nuts therein were discarded. The mesocarp obtained from the fresh oil palm fruitlets (hence known as unsterilised fruitlets) were dried overnight at 60°C. Thereafter, the unsterilised fruitlets were ground to *ca.* 0.3 cm. Mesocarp obtained from sterilised oil palm fruitlets (hence known as sterilised fruitlets) were treated similarly before being further used for extraction.

Influence of Water in DES on Extraction of Ferulic Acid

The influence of water in DES on the extraction of ferulic acid was studied by mixing 0.5 g finely ground sterilised palm fruitlets with neat (0 wt.% water) ChCl-AA (10 mL) in a round bottom flask. The mixture was then heated at 60°C in a tabletop microwave oven (Panasonic NN-CD9975, 900W, 2.4GHz) for 9 min. The heating temperature was controlled by manipulating the microwave power. The mixture was then left to cool to room temperature. Thereafter, the solids were removed from the mixture first by filtration, followed by centrifugation (9000 rpm, 5 min). The supernatant was then dissolved in methanol and the ferulic acid contained therein was determined using high-performance liquid chromatography (HPLC). A similar procedure was used for ChCl-AA containing 10 wt.%, 20 wt.%, 30 wt.%, 40 wt.% and 50 wt.% water. All extractions were carried out in triplicates. The whole experiment was then repeated with ChCl-CA and with unsterilised palm fruitlets, respectively.

Influence of Microwave Heating Temperature

ChCl-AA containing 30 wt.% water (10 mL) was added into a flat bottom flask containing 0.5 g ground sterilised oil palm fruitlets. The mixture was then heated at 40°C for 9 min using microwave. The heating temperature was controlled by manipulating the microwave power. Thereafter, the solids were removed from the mixture first by filtration, followed by centrifugation (9000 rpm, 5 min). The supernatant was then dissolved in methanol and the ferulic acid contained therein was determined using HPLC. Similar procedure was repeated for microwave heating temperatures of 50°C, 60°C, 70°C and 80°C. The whole experiment was then repeated with ChCl-CA and with unsterilised palm fruitlets, respectively. All extractions were carried out in triplicates.

HPLC Analyses of Ferulic Acid

Analyses were carried out according to the method by Ng (2020) with Waters HPLC (e2695 Separations Module) connected to a fluorescence detector (2475 FLR; 321 nm excitation, 418 nm emission). Calibrations of the ferulic acid were carried out with authentic standard.

DPPH Assay of Ferulic Acid Extracts

The ability of ferulic acid extracted from the oil palm fruitlets to scavenge free radicals was determined using DPPH radical scavenging assay outlined by Brand-Williams *et al.* (1995).

Extraction Optimisation (Temperature and Duration)

Optimisations of microwave heating temperature and duration for the extraction of ferulic acid from palm fruitlets were carried out following the procedure outlined above to observe the effect of heating temperature. Sterilised palm fruitlets in ChCl-AA containing 30 wt.% water were heated for 3 to 15 min, at 40°C to 70°C respectively. The experiment was repeated using ground unsterilised palm fruitlets and with ChCl-CA. All extractions were carried out in triplicates.

RESULTS AND DISCUSSION

Influence of Water in DES

DES with ChCl as base is used in this study with CA and AA as hydrogen bond donors (HBD). The higher viscosity of CA (6.50 mm²/s) than AA (1.06 mm²/s) was reflected in their resultant DESs (22.66 ± 0.65 mm²/s for ChCl-CA and 9.68 ± 0.46 mm²/s for ChCl-AA) (Figure 1).

Viscosity is often a limitation faced by DESs as interactions among DESs own components are strong and thus inhibited its infiltration into the substrate, causing low extraction efficiency (Hammond *et al.*, 2017; Wang *et al.*, 2018). The addition of water into DESs will alter their viscosities, which in turn, affect their behaviour (Ng and Nu'man, 2021; 2022a; Wang *et al.*, 2018). In other words, extraction efficiency could be greatly affected by the viscosity or the amount of water in the DES.

Viscosities of ChCl-AA and ChCl-CA decreased rapidly as the amount of water contained therein increased from 0 wt.% to 10 wt.% (22.66 ± 0.65 mm²/s to 10.22 ± 0.65 mm²/s for ChCl-CA and 9.68 ± 0.46 mm²/s to 5.74 ± 0.38 mm²/s for ChCl-AA). This drastic change in viscosity was attributed to the hygroscopic nature of the DESs where even a small amount of water could alter their structures and properties (Cvjetko *et al.*, 2015; Dai *et al.*, 2013a; 2013b; Hammond *et al.*, 2017). The viscosities of both ChCl-CA and ChCl-AA continued to drop with the increasing amount of water within up to 50 wt.%. Although there was a decline in viscosities for both DES with low water content, the drop in viscosities for both DES started to taper as the amount of water within rose beyond 30 wt.% (Figure 1).

Another observation was that although ChCl-AA with or less than 20 wt.% water had significantly lower viscosity than ChCl-CA containing the same amount of water, the difference in viscosity between these two DESs was not significant as the amount of water in the DES increased beyond 30 wt.%. Both ChCl-CA and ChCl-AA containing 50 wt.% water had almost similar viscosity, 1.66 ± 0.36 mm²/s and

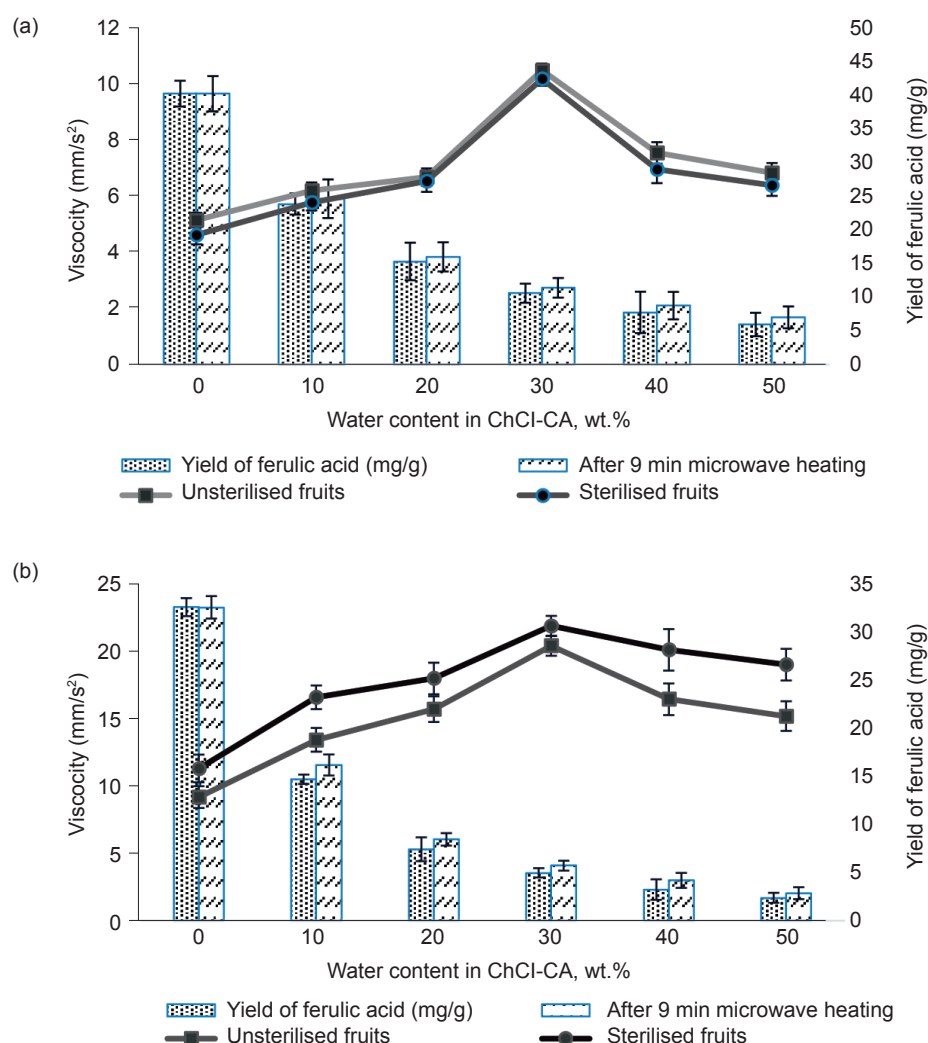


Figure 1. Viscosities of (a) ChCl-AA and (b) ChCl-CA with different amounts of water before and after microwave heating and yield of ferulic acid after 9 min heating at 5°C.

$1.43 \pm 0.42 \text{ mm}^2/\text{s}$ respectively. This was because the DES started to lose its properties and lean towards aqueous behaviour in the presence of a large amount of water as reported in previous studies (Chen *et al.*, 2016; Sharma and Dash, 2021; Wang *et al.*, 2018). ChCl-based DES could tolerate up to 51 wt.% water, beyond which, the DES becomes an aqueous solution instead (Abbott *et al.*, 2005).

In general, moisture could be lost when a solution containing water is heated. Loss of moisture through microwave heating could affect the viscosity and hence the extraction efficiencies of the DES. As such, the viscosities of the DESs without palm fruitlets samples (blank) were recorded both before and after microwave heating to rule out the possibility of the DESs having undergone permanent viscosity changes due to heat treatment. Although there was a slight increase in the viscosities of both ChCl-AA and ChCl-CA after 9 min microwave heating, the changes were not significant, thus showing that

heating did not have any profound effect on the DESs (Figure 1).

A larger amount of ferulic acid could be obtained from unsterilised palm fruitlets when extracted using ChCl-AA than ChCl-CA (Figure 1). However, the difference is not significant for sterilised palm fruitlets except for DES with 30 wt.% water where higher amount of ferulic acid could be obtained using ChCl-AA ($42.5 \pm 1.1 \text{ mg/g}$) than ChCl-CA ($29.8 \pm 1.0 \text{ mg/g}$). This may be due to easier penetration of DES containing certain amount of water into the sample matrices that have been pretreated with heat as were the case with sterilised palm fruitlets and supported by previous studies (Rente *et al.*, 2021; Woo *et al.*, 2015).

Although the difference in viscosity between ChCl-AA and ChCl-CA containing 40 wt.% and 50 wt.% water was not significant, the similarity in viscosities did not translate to similar extraction efficiencies of ferulic acid from unsterilised palm fruitlets. Higher amount of ferulic acid were

obtained from unsterilised palm fruitlets using ChCl-AA than ChCl-CA (28.5 ± 1.5 mg/g to 31.5 ± 1.6 mg/g and 20.7 ± 1.5 mg/g to 29.1 ± 2.1 mg/g respectively) although the viscosities of both DES were quite similar. This indicated that the property or type of hydrogen bond donor (HBD) in DES is more profound than viscosity in the extraction for unsterilised palm fruitlets. However, the type of HBD did not significantly affect the extraction with sterilised palm fruitlets whereby there was not much difference in the amount of ferulic acid extracted with both types of DES containing similar amount of water (26.7 ± 1.6 mg/g to 29.1 ± 2.1 mg/g for ChCl-AA and 25.9 ± 1.6 mg/g to 27.4 ± 2.1 mg/g for ChCl-CA). A possible explanation is that DES with carboxylic acids, such as ChCl-AA, has better extraction efficiencies due to the reaction between the carboxylic functional groups and its base, ChCl (Liu *et al.*, 2019; Rente *et al.*, 2021). This, in turn, led to higher destruction and reconnection inhibition of the cell walls to cellulose, hemicellulose and lignin of the palm fruitlets. However, this process is more profound in unsterilised palm fruitlets which have not undergone any heat treatment compared to sterilised palm fruitlets which have been subjected to high temperatures during sterilisation. The high temperature employed during sterilisation (130°C - 160°C) would have softened the fibre matrices rendering them to be more accessible regardless of the types of HBD present in the DES (Hammond *et al.*, 2017; Ma *et al.*, 2018; Rente *et al.*, 2021).

A general pattern was observed whereby larger amount of ferulic acid could be extracted from palm fruitlets when DES with up to 30 wt.% water was used for extraction. Extraction efficiencies, reflected by the concentrations of ferulic acid, dropped when the amount of water in the DES increased beyond 40 wt.%. This was observed for both types of DESs, regardless of the conditions of the palm fruitlets (unsterilised or sterilised). This could be due to the excessive water content in the DESs where it started to behave as an aqueous solution rather than as DES. This was reported in previous studies whereby the addition of an excessive amount of water into DES disrupted the hydrogen bonds between DES and water, hence reducing the interactions between the extraction medium and target compounds (Hammond *et al.*, 2017; Rente *et al.*, 2021; Wang *et al.*, 2018). This showed that even if extraction efficiency could be increased by changing the viscosity of DESs with water, there remained a limit whereby the DESs could no longer withstand the presence of water without changing their behaviour.

The highest concentrations of ferulic acid were obtained when unsterilised and sterilised palm fruitlets were extracted using ChCl-AA containing 30 wt.% water (43.8 ± 1.0 mg/g and 42.5 ± 1.0 mg/g respectively).

Radical Scavenging Activities of Ferulic Acid Extracts

The ferulic acid extracts obtained from microwave-heated DES with varying water content were tested for a radical scavenging activity to ensure their biochemical activity is not hindered by the DES or the extraction process. Radical scavenging activity of the ferulic acid extracts from unsterilised and sterilised palm fruitlets were determined by DPPH assay with the following Equation (1).

$$\text{Scavenging activity (\%)} = \frac{(A_0 - A_1) \times 100\%}{A_0} \quad (1)$$

where A_0 is the absorbance of control and, A_1 is the absorbance of extract.

Ferulic acid extracted from both types of oil palm fruitlets had good radical scavenging activity ranging from 88%-95% (Figure 2). Although sterilisation during palm oil milling did not have a significant effect on the content of ferulic acid whereby the difference in the amount of ferulic acid obtained from unsterilised and sterilised palm fruitlets was not significant, it caused a reduction in its antioxidative power whereby unsterilised fruitlets extracts exhibited higher radical scavenging activity compared to sterilised fruitlets extracts (92% to 95% and 88% to 92% respectively). While there was not much difference in the radical scavenging activity in unsterilised fruitlets extracts, the activity in sterilised fruit extracts seemed to increase with the escalation in the amount of water in the DES used for extraction to up to 30 wt.%. The difference in radical scavenging activity in sterilised fruitlets extracts was not significant in extracts obtained via DESs containing more than 30 wt.% water. A possible explanation for this is that the presence of water affected the extraction process as well as its physicochemical properties, which was also reported in the findings of previous study (Yuan *et al.*, 2005).

Taking in concentrations as well as radical scavenging activities of ferulic acid in the extracts obtained via DES with different water content, it was perceived that DES containing 30 wt.% water was the best medium to extract ferulic acid from palm fruitlets. As such, similar DESs were used to further study the effect of heating temperature and duration of microwave irradiation on ferulic acid extraction.

Effect of Microwave Heating Temperature

Generally, higher extraction temperature leads to higher mass transfer between substrate and extraction medium (Abbott *et al.*, 2004; Bi *et al.*, 2013; Ng and Nu'man, 2022a). Studies on the effect of microwave heating temperature on the extraction of

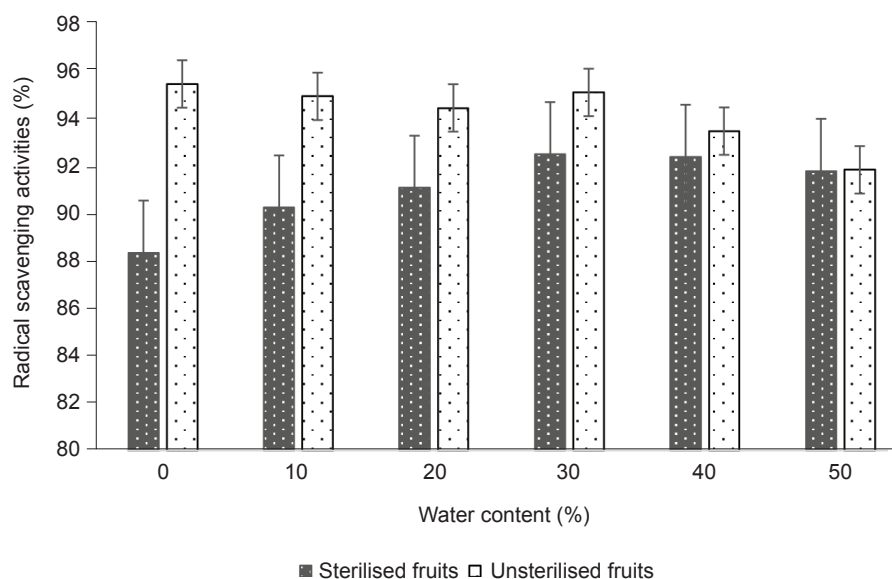


Figure 2. Free radical scavenging activities of ferulic acid extracted from oil palm fruitlets using DES with microwave heating.

ferulic acid from palm fruitlets using ChCl-CA and ChCl-AA were carried out whereby the temperature of the microwave heating increased by 10°C with each increment, beginning with 40°C to 80°C while the heating duration was held constant. While palm fruitlets were charred at 80°C, the results of the rest of the study are depicted in Figures 3 and 4.

At low temperature (40°C), the efficiencies for both DESs to extract ferulic acid from both palm fruitlets were low (Figures 3 and 4). Although theoretically, high temperature could lead to higher extraction efficiencies, it is also important to keep in mind that most antioxidative compounds are sensitive to heat. Degradation of ferulic acid generally began at 40°C, indicating that high temperature is not suitable for its extraction (Das and Wong, 2020; Ng and Nu'man, 2021). Nevertheless, it is worth investigating the temperature where real and significant damage occurs.

As the extraction temperature increased to 50°C and subsequently 60°C, the amount of ferulic acid obtained from the reaction of sterilised fruitlets with both types of DESs was significantly higher than each previous extraction temperature. High temperature reduced the physical and chemical interactions between ferulic acid and fruitlets matrices resulting in better penetration of the DES, thus accelerating the mass transfer of ferulic acid from the fruitlets to the DES. However, as the extraction temperature escalated to 70°C, drop in the concentrations of ferulic acid was observed. Similar pattern was reported for unsterilised fruitlets in ChCl-CA. However, when unsterilised fruitlets were reacted with ChCl-AA, the drop in the amount of ferulic acid obtained occurred at lower temperature, 60°C instead of 70°C as in the case for ChCl-CA.

Larger amount of ferulic acid was extracted from both types of fruitlets using ChCl-CA at 60°C than 70°C. The highest concentrations of ferulic acid obtained via ChCl-CA from unsterilised and sterilised fruitlets were 38.1 ± 1.2 mg/g and 37.2 ± 1.2 mg/g respectively, which occurred at 60°C after 6 min heating. These were slightly less than what could be obtained with ChCl-AA as extraction medium, whereby the highest concentration of ferulic acid extracted from sterilised fruitlets was 46.9 ± 1.2 mg/g, under the same extraction conditions as ChCl-CA (6 min heating at 60°C). However, lower temperature was more conducive for the extraction of ferulic acid from unsterilised palm fruitlets using ChCl-AA whereby the highest ferulic acid concentration (43.8 ± 1 mg/g) was obtained at 50°C after 9 min heating.

Effect of Microwave Irradiation Duration

Microwave irradiation rapidly increases the temperature which enabled high extraction efficiency of heat sensitive compounds in a short period of time (Wang *et al.*, 2018; Xie *et al.*, 2019). Extraction of ferulic acid from palm fruitlets with DES could be accomplished in as little as 3 min of microwave heating at 40°C. Extending the microwave heating duration at 40°C from 3 to 15 min increased the concentration of ferulic acid extracted from unsterilised fruitlets via ChCl-CA (14.2 ± 0.7 mg/g to 20.0 ± 0.9 mg/g) and ChCl-AA (18.2 ± 0.7 mg/g to 29.4 ± 0.9 mg/g). Similar increase was also observed for sterilised fruitlets reacted with ChCl-CA (12.1 ± 0.7 mg/g to 19.2 ± 0.9 mg/g) and ChCl-AA (13.5 ± 0.7 mg/g to 24.1 ± 0.9 mg/g) under the same conditions (Figures 5 and 6).

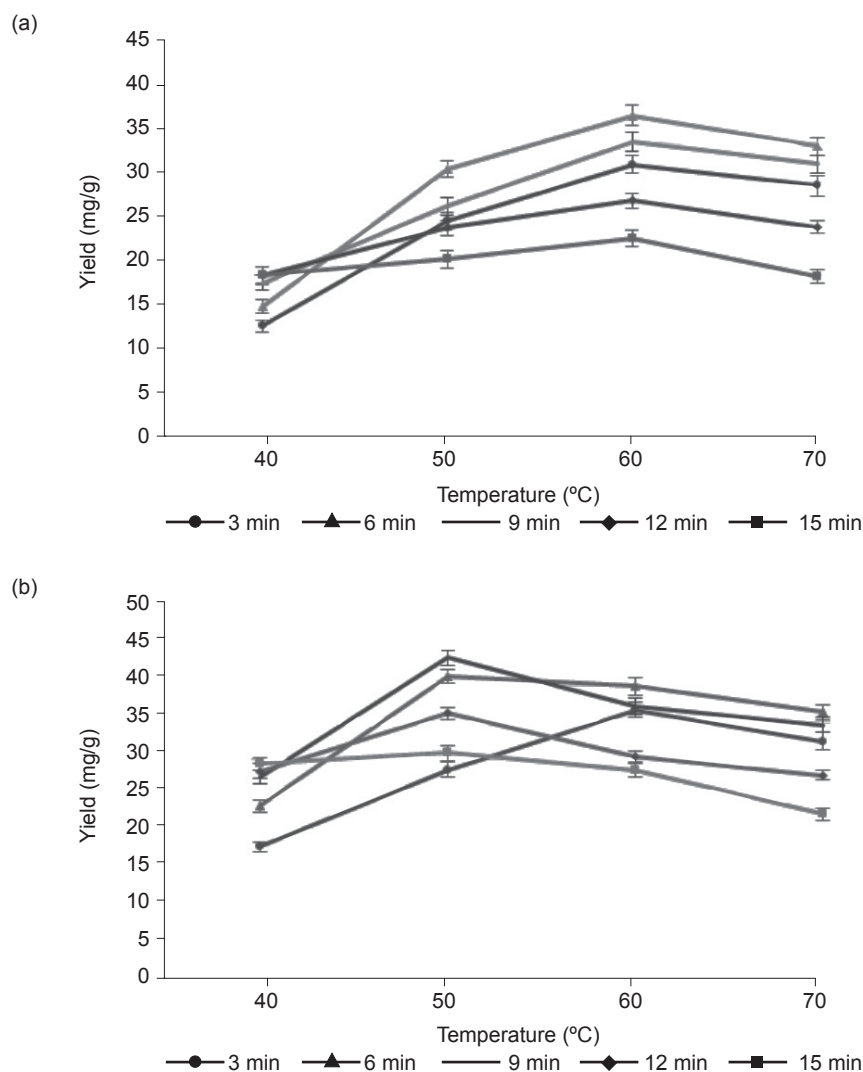


Figure 3. Yield of ferulic acid extracted from unsterilised fruitlets using (a) ChCl-CA and (b) ChCl-AA containing 30 wt.% water at different heating duration and temperatures.

Except for extractions at low temperature (40°C), both sterilised and unsterilised fruitlets could not withstand more than 6 min microwave heating in ChCl-CA at all heating temperatures studied (Figures 5 and 6). Initially, extending the heating duration for both types of palm fruitlets in ChCl-CA from 3 to 6 min had a positive impact whereby the amount of ferulic acid obtained increased with the increase in heating duration (Figures 5 and 6). However, as the heating duration was further extended, drop in the ferulic acid yield was observed. By the end of 15 min heating at 60°C, the concentrations of ferulic acid in unsterilised and sterilised fruitlets extracts (24.1 ± 0.9 mg/g and 23.2 ± 0.9 mg/g respectively) were less than their concentrations in extracts obtained via heating for only 3 min (32.5 ± 1.0 mg/g and 31.2 ± 1.0 mg/g respectively) at the same temperature.

Similar pattern was observed for both types of fruitlets in ChCl-AA at 70°C for up to 15 min whereby the concentration of ferulic acid obtained

at the end of 15 min heating was less than after only 3 min heating. Although drop in ferulic acid concentration was observed for sterilised fruitlets extracted beyond 6 min heating at 60°C in ChCl-AA, its concentration by the end of 15 min heating (34.2 ± 0.9 mg/g) at the same temperature was still significantly higher than when it was heated for only 3 min (24.1 ± 0.9 mg/g). This was not the case when ChCl-CA was used as extraction medium. As opposed to ChCl-CA which saw the drop in ferulic acid concentration beyond 6 min heating at 50°C, 60°C and 70°C respectively, the reaction with ChCl-AA could withstand heating slightly longer at 50°C, to up to 9 min heating before drop in ferulic acid's concentrations was observed. The drop in the ferulic acid concentrations was probably due to degradation for being exposed to high temperatures for an extended period as reported by Ng and Nu'man (2021). This indicated that while high temperature could increase extraction efficiency, heat exposure under microwave for

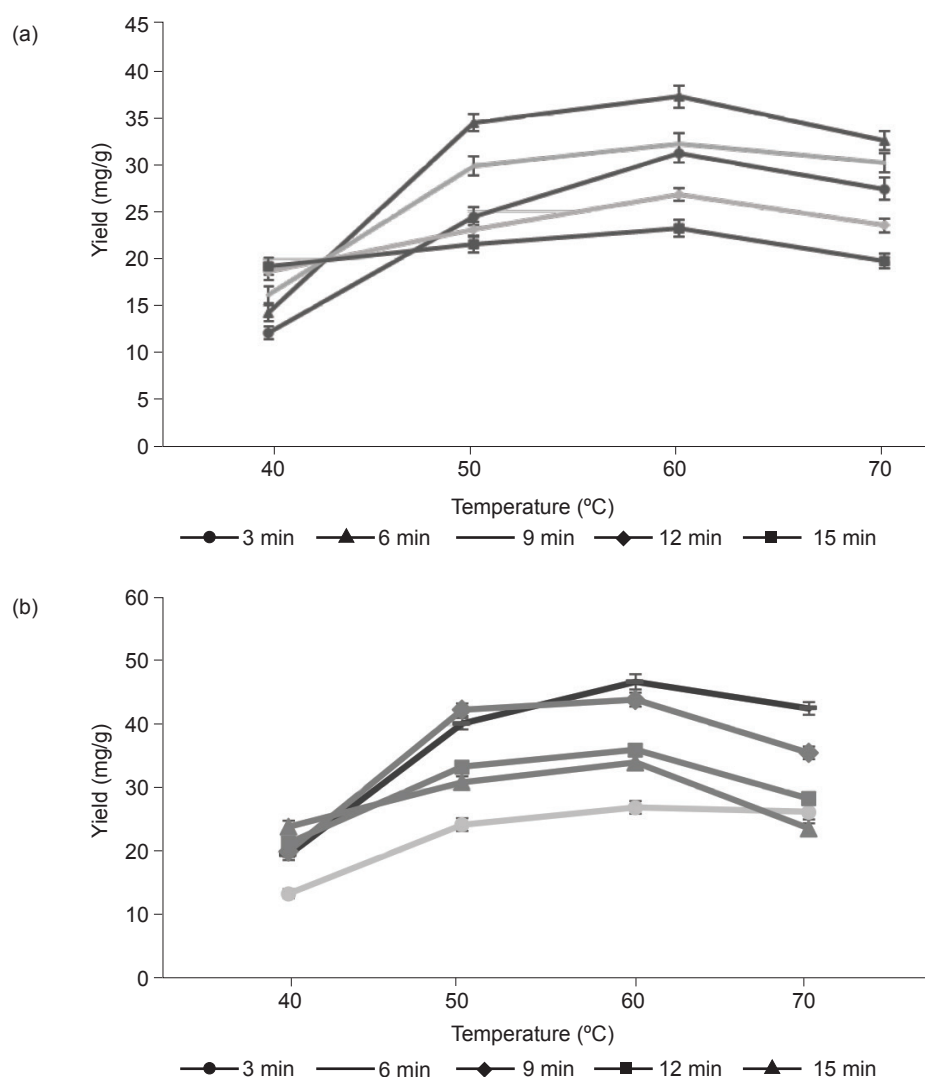


Figure 4. Yield of ferulic acid extracted from sterilised fruitlets using (a) ChCl-CA and (b) ChCl-AA containing 30 wt.% water at different heating duration and temperatures.

a long duration is not advisable. Compared to the conventional extraction method by reflux, microwave-assisted extraction could achieve similar results in a much shorter time (Ng and Nu'man, 2021; 2022a). It was therefore concluded that the optimal conditions for the extraction of ferulic acid from oil palm fruitlets were microwave heating at 60°C for 6 min in DES containing 30 wt.% water (Figures 7 and 8).

Advantages of MAE DES over Other Extraction Methods

Ng (2020) reported the extraction of 0.250 mg/g and 0.668 mg/g ferulic acid from unsterilised and sterilised palm fruitlets respectively through 6 hr methanolysis. In comparison, *ca.* 46.900 mg/g of ferulic acid was obtained from this study, which was significantly higher than that achieved by Ng (2020). This could be due to efficient desolvation of lignin in palm fruitlets by DES under microwave

influence, which then facilitated the transfer of ferulic acid from the fruit matrices to the DES. The high extraction efficiency was also contributed by lower DES viscosities as a result of the addition of water into the DES therein which is among the determining factor in the extraction of phenolic compounds (Dai *et al.*, 2013a; Liu *et al.*, 2019).

Through alkaline hydrolysis, Mohd Aanifah *et al.* (2014) reported the recovery of 3800-4200 ppm of ferulic acid from palm empty fruit bunch. Besides alkali, hydrolysis catalysed by cutinase had also been reported as a way to extract ferulic acid from palm empty bunches (Tang *et al.*, 2015) in which *ca.* 0.050 mg/g ferulic acid was obtained after 24 hr reaction. Oxidation of palm empty fruit bunches with nitrobenzene in 10 wt.% sodium hydroxide resulted in the extraction of *ca.* 0.045 mg/g ferulic acid in a reaction that took 180 min. However, nitrobenzene oxidation is severe and thus, could not protect the ferulic acid from degradation.

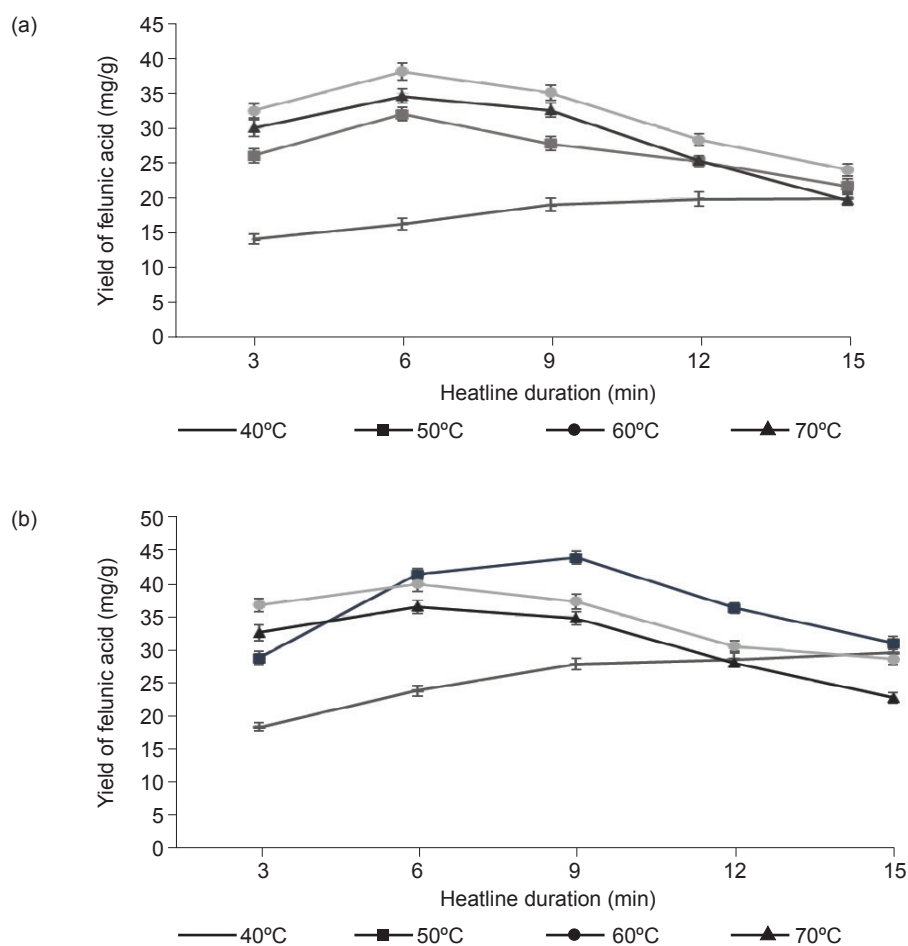


Figure 5. Yield of ferulic acid extracted from unsterilised fruitlets using (a) ChCl-CA and (b) ChCl-AA containing 30 wt.% water at different heating duration and temperature.

Wang *et al.* (2018) reported the advantages offered by microwave heating whereby dissolution and diffusion rate of baicalin from plant cells occurred at a much shorter time with microwave compared to conventional heating. Ng and Nu'man (2021) reported a yield of 0.609 - 0.617 mg/g and up to 1.123 mg/g ferulic acid when palm pressed fibre was extracted with DES under 15 min microwave heating. In another study with extraction conducted using ChCl-AA by reflux, it was reported that the optimal condition for the extraction of ferulic acid from palm pressed fibre was 6 hr heating, which was much longer compared to extractions carried out by microwave heating (Ng and Nu'man, 2022a). This shows that a combination of microwave heating with DES could greatly enhance extraction efficiency in a much shorter reaction duration. DES and microwave could complement each other by offering homogenous heating at high speed and efficiency, accelerating plant cell rupture and releasing intracellular compounds therein, resulting in superior extraction efficiency in a shorter duration.

Besides hydrolysis and microwave heating, other assisted techniques to extract ferulic acid from palm include the use of ultrasonic whereby oil palm biomass was reacted with acidified ethanol in an

ultrasonic bath. As much as 0.560 mg/g of ferulic acid could be extracted from palm kernel cake after 40 min through this technique (Tsouko *et al.*, 2019). However, there was no mention of the amount of ferulic acid extracted from palm kernel shells, empty bunches, as well as pressed mesocarp fibre of the oil palm. Considering both the time required for completion of extraction as well as the amount of ferulic acid that could be obtained, DES extraction with microwave heating is a superior technique for the extraction of ferulic acid compared to other reported methods.

CONCLUSION

Extraction of ferulic acid from both unsterilised and sterilised palm fruitlets by DES could be assisted with microwave heating whereby the extraction duration could be drastically reduced compared to conventional extraction techniques. AA is a better HBD than CA in choline chloride-based DES for the extraction of ferulic acid whereby higher concentrations of ferulic acid were obtained with ChCl-AA as the extraction medium. The optimal conditions for the extraction of ferulic acid from oil

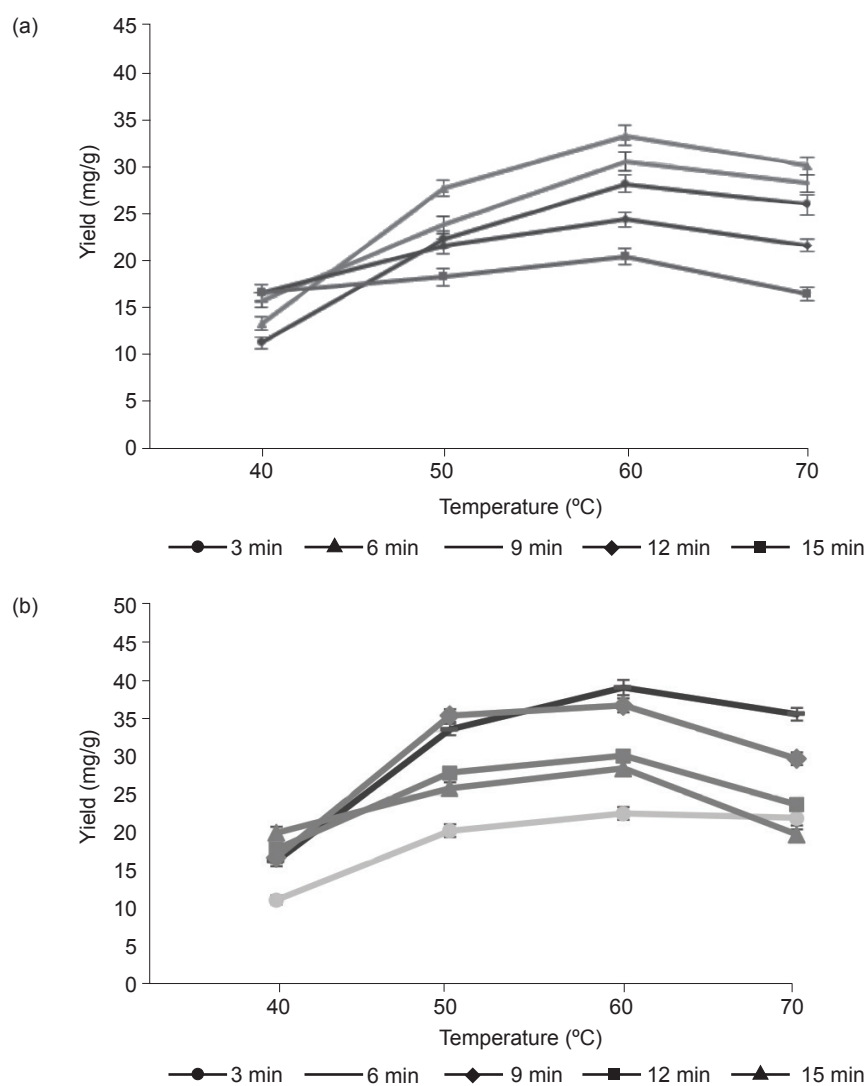
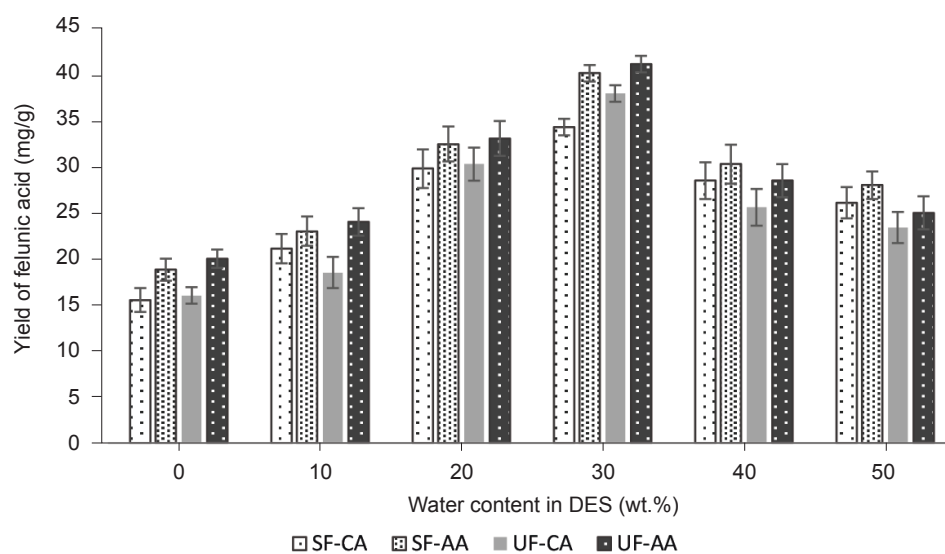
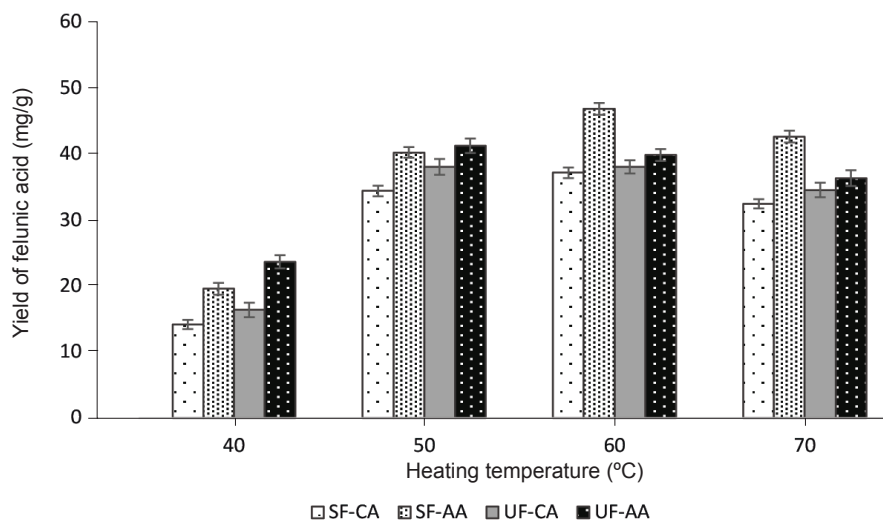


Figure 6. Yield of ferulic acid extracted from sterilised fruitlets using (a) ChCl-CA and (b) ChCl-AA containing 30 wt.% water at different heating duration and temperature.



Note: SF-CA - Sterilised fruits extracted with ChCl-CA; SF-AA - Sterilised fruits extracted with ChCl-AA; UF-CA - Unsterilised fruits extracted with ChCl-CA; UF-AA - Unsterilised fruits extracted with ChCl-AA.

Figure 7. Yield of ferulic acid extracted with DES containing varying water content after 6 min heating at 50°C.



Note: SF-CA - Sterilised fruits extracted with ChCl-CA; SF-AA - Sterilised fruits extracted with ChCl-AA; UF-CA - Unsterilised fruits extracted with ChCl-CA; UF-AA - Unsterilised fruits extracted with ChCl-AA.

Figure 8. Yield of ferulic acid extracted with DES containing 30 wt.% water and 6 min microwave heating under varying temperature.

palm fruitlets were microwave heating at 60°C for 6 min with DES containing 30 wt.% water. Sterilisation during palm oil milling did not significantly affect the amount of ferulic acid in the oil palm fruitlets as proven in this study. However, ferulic acid extracts from sterilised fruitlets had slightly lower antioxidative activities than unsterilised fruitlets extracts.

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COMPARATIVE STUDIES ON CURE CHARACTERISTICS AND MECHANICAL PROPERTIES OF OIL PALM BIOMASS FILLED NATURAL RUBBER COMPOSITES

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and Z NAHRUL HAYAWIN^{3*}

ABSTRACT

Oil palm is categorised as the most important agricultural crop in Malaysia occupying about 70% of the agricultural land. The palm oil industry is one of the biggest contributors to Malaysia's Gross National Income (GNI). However, the rapid development of the palm oil industry causes high production of palm oil waste which is a worrying environmental issue these days. As the need to promote sustainable, renewable and clean energy keeps rising, researchers have taken a keen interest in utilising palm oil waste as a source of biomass. Palm oil waste such as palm kernel shell (PKS) is a great source of valuable biomass and readily available in large amounts which can then be transformed into biochar and activated carbon via pyrolysis and activation processes, respectively. By producing palm kernel shell biochar (PKSB) and activated palm kernel shell (APKS) derived from palm oil waste, they can potentially be used as potential bio-fillers in rubber composites. The research aimed to study the effect of different loading of PKSB and APKS in natural rubber (NR) composite since they have higher carbon content, porosity and surface area compared to other biomass fillers. The surface morphology analysis by scanning electron microscopy, cure characteristics, swelling measurements, and mechanical properties was analysed. APKS was observed to have well-developed pores that could provide a high surface area of contact resulting in improved mechanical interlocking between filler and rubber matrix. Comparisons were made between PKSB, APKS and other bio-fillers such as coconut fibre (CF), bamboo charcoal (BC) and untreated kenaf (UK). The cure characteristics for PKSB showed a high range of maximum torque (M_H) values (11-23 dN.m), better in ts_2 (1 min) and t_{90} (2-3 min), whereas the M_H values for APKS were slightly lower and longer in t_{90} which were 14-15 dN.m and 2-4 min, respectively. PKSB also showed a better swelling percentage (226%-329%) which would indicate high crosslink density compared to APKS (305%-336%). Moreover, APKS showed better tensile strength (8-18 MPa) and elongation at break (519%-669%) as the filler loading increased in comparison with PKSB which recorded a tensile strength of 5-14 MPa and elongation at break of 385%-561%. Based on the results, it can be concluded that the optimum value of APKS filler loading ratio in NR composite is 40 phr as 40 phr of APKS filled NR composite had the highest mechanical properties compared to the other filler loading ratios. Both PKSB and APKS have great potential to be used and commercialised as semi-reinforcing fillers in the rubber industry. This way, PSKB and APKS are able to provide a sustainable alternative to existing petroleum-based fillers and could help to reduce the abundance of palm oil waste while producing high-performance rubber-based products.

Keywords: activated carbon, biochar, cure characteristic, mechanical properties, palm kernel shell.

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INTRODUCTION

Palm oil production has increased rapidly over the past 50 years (Basiron, 2007). According to the Malaysian Palm Oil Council (MPOC), Malaysia is among the world's largest palm oil producers, generating approximately 19.14 million tonnes and exporting 17.37 million tonnes of palm oil, respectively in 2020. However, these figures were lower than in 2019 due to the COVID-19 pandemic and the government's movement control order (MCO) (Parveez *et al.*, 2020). Massive palm oil production generates large amounts of oil palm waste, causing disposal issues, pollution and global warming effects if not handled properly (Abbas *et al.*, 2019). Oil palm trunks (OPT), oil palm fronds (OPF), empty fruit bunches (EFB), mesocarp fibres (MF), palm kernel shell (PKS) and palm oil mill effluent (POME) are the waste biomasses that are produced from the palm oil industry. However, these palm oil biomasses can be converted into useful applications such as potential fillers in polymer composites to curb these environmental problems. Among these palm oil biomass, PKS can be transformed into biochar and activated carbon as fillers due to its physico-chemical properties in terms of porous surface, high mechanical strength, insolubility in water, and various functional groups (Anisuzzaman *et al.*, 2021).

Fillers are compounding ingredients used in rubber compounding to reinforce or reduce the cost of polymeric material (Abidin *et al.*, 2020; Egwaikhide *et al.*, 2007; Mamaud *et al.*, 2018). Carbon black (CB) and silica are the most widely used conventional fillers in the rubber industry because of their ability to improve particular mechanical qualities and their high availability (Moonchai *et al.*, 2012). Biomass resources such as bamboo, kenaf, cellulose, starch, coconut fibre, rice husk and palm kernel shell are frequently employed in rubber composites as alternative bio-fillers (Arayaprane *et al.*, 2005; Egwaikhide *et al.*, 2007; Ismail *et al.*, 2010; Onyeagoro, 2012). Alternative bio-fillers from these biomasses have numerous advantages over conventional fillers as they are inexpensive, possess low density, are easily biodegradable and have a low environmental

impact (Daud *et al.*, 2017; Hidayu and Muda, 2016; Onoja *et al.*, 2019). Many researchers have investigated the use of PKS as fillers in rubber compounds due to their biomaterial compatibility with interesting properties such as porosity, water content, lignocellulosic properties, solid density and carbon content (Baffour-Awuah *et al.*, 2021). However, there are some weaknesses in PKS such as having low or blocked porous structure due to the presence of contaminants and a low percentage of carbon content that could limit its performance as a bio-filler for rubber composites.

Thus, the carbonisation and activation process are applied to PKS at high temperatures to improve the weaknesses of PKS. The carbonisation process is a thermal decomposition of organic material in the absence of oxygen which can eliminate any volatile matter, develop a porous structure and increase the carbon content (Rugayah *et al.*, 2014). It also can create mechanical interlocking between filler and rubber matrix with the presence of their porous structure hence improving the mechanical strength of the composites. Whereas, the carbonisation-activation process is a two-step process in which the material is carbonised at a temperature of around 500°C and activated at a temperature of around 900°C. This process uses higher heating temperatures compared to the carbonisation process. With this higher heating temperature, it has the ability to enhance the properties of palm kernel shells better in terms of carbon content, surface area and development of pores structure (Hamza *et al.*, 2015).

Nowadays, many renewable and natural fillers such as rice husk, coconut shell and bamboo have been extensively used in rubber-based applications. One of the renewable and natural fillers sources would be PKS and it has the potential to be used as a bio-filler in the rubber industry due to its high lignin content, along with hemicellulose that could give the fibre toughness and stiffness (Daud *et al.*, 2016). PKS also has the ability to be transformed into an enhanced version of bio-filler known as biochar and activated carbon by undergoing carbonisation and activation processes. These processes could improve their specific desirable features such as creating porous structure and increasing the carbon content, which are very important features as part of rubber reinforcement (Farida *et al.*, 2019). Previous studies involving palm kernel shell biochar (PKSB) and activated palm kernel shell (APKS) as filler reinforcement in rubber matrices are quite low in number due to a lack of research about it. This study involves the utilisation of PKSB and APKS of high carbon content and porosity as reinforcement in NR composites. Both PKSB and APKS have great, promising potential to be used

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as a semi-reinforcing filler in NR composite. This study could encourage rubber researchers around the world to transform biomass PKS into a value-added additive and incorporate it into rubber matrices. Therefore, this study aimed to analyse the potential and performance of both PKSB and APKS as bio-fillers in natural rubber (NR) by adding different filler loading in comparison with other commercial fillers. The effects of PKSB and APKS on the morphology, cure characteristics and mechanical properties of NR composites were investigated by using a scanning electron microscope (SEM), swelling measurements and tensile test.

MATERIALS AND METHODS

Preparation of Materials

Natural rubber bale (grade SMR-10) was supplied by a local company, Airelastic Industries Sdn. Bhd. while the raw PKS was obtained from the Ulu Kanchong Palm Oil Mill, Ulu Kanchong Estate, Negeri Sembilan, Malaysia. PKS was converted into PKSB and APKS by carbonisation and activation processes following the method from previous studies (Nahrul Hayawin *et al.*, 2017; 2020). This is shown in *Figures 1a, 1b* and *1c*. PKS underwent a carbonisation process at 500.0°C where approximately 40 kg of PKS was fed into a rotary kiln and the process took around 60 min to form PKSB (Mohd Hasan *et al.*, 2019; Nahrul Hayawin *et al.*, 2020). The PKSB was activated using a 2-in-1 carbonisation activation process at 900.0°C to produce APKS (Nahrul Hayawin *et al.*, 2020). The PKSB and APKS were first washed using distilled water to remove any contaminants and the pH value was checked by using a pH meter until pH 7 was obtained and further dried

in an oven for 24 hr at 104.5°C. Since acidity tends to diminish the crosslink density of filler, resulting in a slow vulcanisation cure rate, the pH of the samples must be neutral (Malomo *et al.*, 2020). Both PKSB and APKS were then pulverised into powder form which could be seen in *Figure 1c* and sieved via a vibratory sieve shaker with a mesh size of 240.

Preparation of PKSB- and APKS-filled NR Composites

Figure 2 shows the experimental design of PKSB and APKS as fillers in NR composites. It shows the process starting from the morphological study of PKSB and APKS particles until mechanical testing was conducted. *Table 1* shows the formulation of rubber compounding used in this study, which were F1 (without filler), F2-F7 (PKSB filler) and F8-F13 (APKS filler). The NR (SMR-10) was incorporated in a two-roll laboratory mill at room temperature according to ASTM D3184. The roll was preheated for 1 min, then the rubber was placed into the mill and pre-masticated until it became soft and formed a band around the mill. Following the pre-mastication procedure, the ingredients were added in the following order; zinc oxide, stearic acid, PKSB or APKS, 2,2,4-trimethyl-1,2-dihydroquinoline (TMQ), 2,2'-dithiobisbenzothiazole (MBTS), 1,3-diphenylguanidine (DPG), tetramethylthiuram disulfide (TMTD), paraffin oil and sulphur. All compounding ingredients were mixed for about 10-15 min. The finalised mixtures were sheeted to about 3 mm thick and cooled for 24 hr at room temperature. The mixtures were then compressed into rubber composites via placement in a mould and pressed between heated plates of the hot press machine at 160°C for curing. The amount of time used for the compression was based on the results of curing characteristics in *Table 4*.



Figure 1. (a) Palm kernel shell biochar (PKSB), (b) activated palm kernel shell (APKS) and (c) fine PKSB and APKS powder after pulverised.

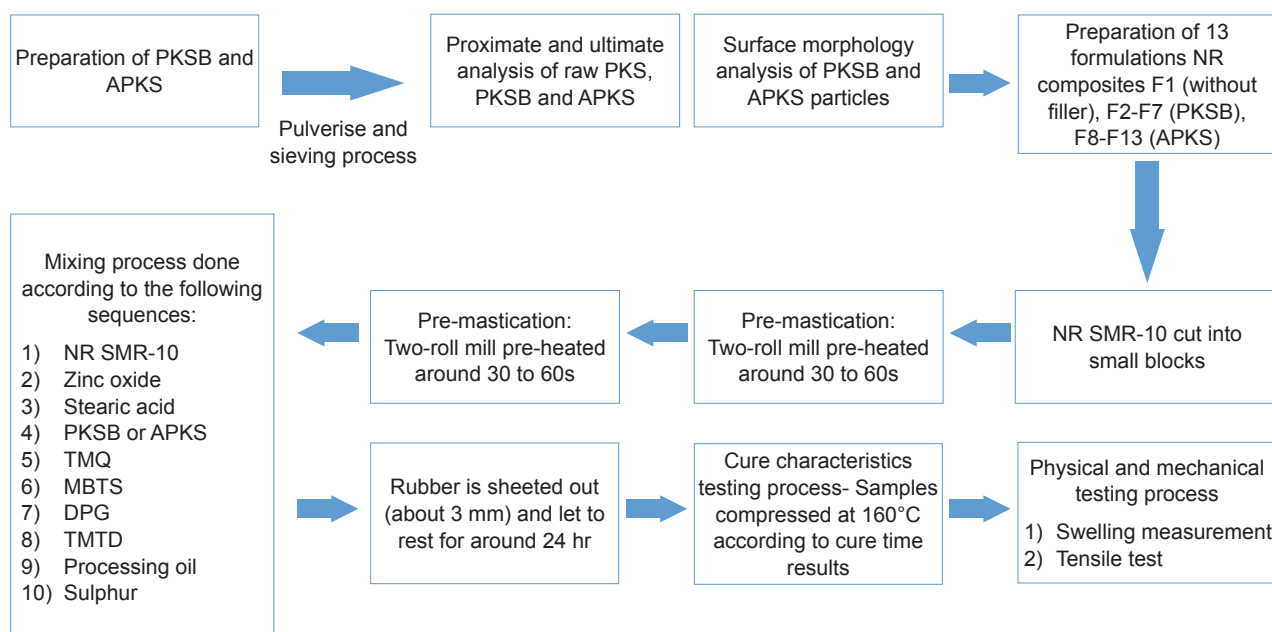


Figure 2. Experimental design of PKSB- and APKS-filled NR composites.

TABLE 1. RUBBER COMPOUNDING FORMULATIONS

Ingredients (phr)	Formulation												
	1	2	3	4	5	6	7	8	9	10	11	12	13
NR	100	100	100	100	100	100	100	100	100	100	100	100	100
PKSB	0.0	5.0	10.0	20.0	30.0	40.0	50.0						
APKS								5.0	10.0	20.0	30.0	40.0	50.0
Stearic acid	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Zinc oxide	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
TMQ	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Oil	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	50	5.0	5.0	5.0	5.0
DPG	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Sulphur	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
TMTD	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
MBTS	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25

Characterisation of PKSB, APKS, PKSB- and APKS-filled NR Composites

Ultimate and proximate analysis. The elemental components which consist of carbon, hydrogen, nitrogen and sulphur content in raw PKS, PKSB and APKS were analysed using a CHNS analyser (LECO, CHNS932, USA) according to the ASTM D5291 method. The proximate analysis was done using a thermogravimetric analyser (TGA) Mettler Toledo, TGA/SDTA 851 (USA) according to ASTM D5142 to determine moisture content, volatile matter, fixed carbon and ash content of raw PKS, PKSB and APKS particles (Nahrul Hayawin *et al.*, 2017).

Morphological study. The surface morphology of PKSB and APKS were analysed and characterised using a scanning electron microscope (Leica Cambridge S-360 SEM) to determine the porous structure and shape of the pores for PKSB and APKS.

Cure characteristics. The PKSB-filled rubber and APKS-filled NR composites were cut between 8-10 g and cure assessment was carried out at 160°C within a test period of about 30 min using a ZME-1005A rubber rheometer ZME tester. Cure time (t_{90}), maximum torque (M_H), minimum torque (M_L), and scorch time (t_{s2}) were obtained from the torque

versus time rheographs. The delta torque (ΔM) of the vulcanised rubber composites was calculated by using Equation (1):

$$\Delta M = M_H - M_L \quad (1)$$

Swelling measurement. The swelling test was performed in toluene to determine the rubber-filler interaction and degree of swelling for the PKSB- and APKS-filled NR composites according to ASTM D3616. The cured rubber sheet was cut into two pieces with a dimension of 1×1 cm and the initial weight of each sample was recorded. The sample was immersed in toluene for 72 hr at room temperature. Then, the swollen sample was removed from toluene after 72 hr, wiped and weighed again until it reached equilibrium weight. The degree of swelling (Q) and the volume fraction of rubber (V_r) were determined using Equations (2) and (3), respectively:

$$Q (\%) = \frac{W_2(g) - W_1(g)}{W_2(g)} \times 100 \quad (2)$$

where W_1 is the weight of the un-swelled composite and W_2 is the swollen weight.

$$V_r = \frac{V_R}{(V_R + V_S)} \quad (3)$$

where V_R is the volume of rubber and V_S is the molar volume of the solvent (toluene).

Tensile test. The tensile test on the PKSB- and APKS-filled NR composites was carried out using a dumbbell-shaped test piece with a width of 4.051 mm and the thickness of the samples was measured. The tensile properties were determined according to ASTM D412 using Instron Universal tensile testing machine (model 5569) at a speed rate of 500 mm/min. The test was repeated 5 times for each formulation and the average value was calculated. The tensile properties such as tensile strength, elongation at break and modulus at 100 and 300 were generated by the software. The reinforcement index of PKSB- and APKS-filled NR composites were calculated using Equation (4):

$$\text{Reinforcement index (RI)} = \frac{\text{Modulus 300 (M300)}}{\text{Modulus 100 (M100)}} \quad (4)$$

RESULTS AND DISCUSSION

Ultimate and Proximate Analysis

Table 2 shows the elemental constituents of raw PKS, PKSB and APKS obtained from the ultimate analysis. The carbon content (C) of PKSB and APKS increased while a decrease in hydrogen (H), nitrogen (N) and sulphur (S) contents were recorded in comparison with raw PKS. This might be due to the high temperature applied for the carbonisation process in PKSB at 500°C and the carbonisation-activation process in APKS at 900°C. This also could be associated with the release of volatile materials in PKSB and APKS that did not affect the carbon content (Hamza *et al.*, 2015). The release of the hydroxyl group may be responsible for the decrease in H content, while the thermal decomposition of lignocellulose components such as lignin, cellulose and hemicellulose may have contributed to the decrease in the N and S contents (Zainal *et al.*, 2018).

The results of the proximate analysis on raw PKS, PKSB and APKS are shown in Table 3. The moisture content of PKSB and APKS decreased as the temperature of carbonisation increased due to the release of the hydroxyl group (-OH) on the surface of raw PKS when high heat was applied (Rugayah *et al.*, 2014). The same trend could also be seen for the volatile matters of PKSB and APKS, where the thermal decomposition in raw PKS released more volatile materials when the temperature increased (Nahrul Hayawin *et al.*, 2017). For fixed carbon, PKSB and APKS showed increasing values compared to raw PKS because both particles had undergone carbonisation and carbonisation-activation processes at high temperatures, which produced more C-C bonds, resulting in the formation of highly carbonaceous molecules (Mohd Hasan *et al.*, 2019; Sallau *et al.*, 2021). The ash content of PKSB and APKS also increased compared to PKS. Previous studies have stated that the increase in the ash content might be related to the increase in pH value or indicate low purity of the carbon material (Mohd Hasan *et al.*, 2019; Rugayah *et al.*, 2014).

TABLE 2. ULTIMATE ANALYSIS OF RAW PKS, PKSB AND APKS

Sample	Carbon, C (%)	Hydrogen, H (%)	Nitrogen, N (%)	Sulphur, S (%)
Raw PKS	60.90 ± 0.20	12.76 ± 0.02	0.66 ± 0.33	0.19 ± 0.01
PKSB	69.20 ± 0.10	3.30 ± 0.08	0.61 ± 0.01	0.04 ± 0.00
APKS	74.38 ± 2.19	2.03 ± 0.24	0.33 ± 0.01	-

TABLE 3. PROXIMATE ANALYSIS OF RAW PKS, PKSB AND APKS

Sample	Moisture content (wt.%)	Volatile matter (wt.%)	Fixed carbon (wt.%)	Ash (wt.%)
Raw PKS	9.55 ± 0.40	53.38 ± 0.21	18.84 ± 0.02	0.87 ± 0.01
PKSB	6.01 ± 0.10	13.08 ± 1.00	75.02 ± 3.30	4.61 ± 2.50
APKS	5.72 ± 0.98	9.35 ± 3.50	80.21 ± 2.00	2.72 ± 0.97

Morphological Properties of PKSB and APKS

The SEM images of PKSB and APKS particles were observed at different magnifications of 1000 $\times$, 2000 $\times$ and 3000 $\times$ as shown in Figure 3. Figures 3a and 3b show that PKSB has many large pores on the surface of the primary particle due to the pyrolysis process that caused the organic material around the particles to decompose. The gradual decomposition of organic materials such as hemicellulose, cellulose and lignin inside PKSB particles may be responsible for the increase in surface and pore volume at 500 $^{\circ}$ C (Wang *et al.*, 2019; Zhao *et al.*, 2017). The surface of PKSB also looked slightly rough with irregular pore sizes ranging from 11 to 133 m²/g that developed due to the loss of volatile substances after the carbonisation at high temperatures (Hamza *et al.*, 2015).

However, the morphology of APKS particles was quite different compared to PKSB since APKS particles have very tiny and more-developed pores as well as rougher surfaces as shown in Figures 3d and 3e. The changes in morphology and formation of rough surfaces of APKS may be attributed to the widened pores created during the physical activation

and the carbonisation process at 900 $^{\circ}$ C (Malomo *et al.*, 2020). Since APKS has more porous structure, this resulted in more surface area of contact for rubber-filler interaction and promoted mechanical interlocking between APKS and NR phases. The surface roughness observed on the surface of APKS also contributed to the enhanced adhesion of APKS towards NR rubber chains (Daud *et al.*, 2017). This stronger rubber-filler adhesion led to a higher tensile strength of the composite, which helped to enhance the properties of composites. Besides that, both PKSB and APKS had irregular shapes of the primary particle as seen in Figures 3c and 3f which could be due to the mechanical crushing process using a pulveriser (Nabil *et al.*, 2013).

Cure Characteristics

Table 4 summarises the cure characteristics of rubber composites without the presence of filler (F1) which acts as a control, PKSB filler (F2-F7) and APKS filler (F8-F13). M_H and M_L evaluate the viscosity of the rubber composites to indicate its processability while ΔM measures the stiffness and

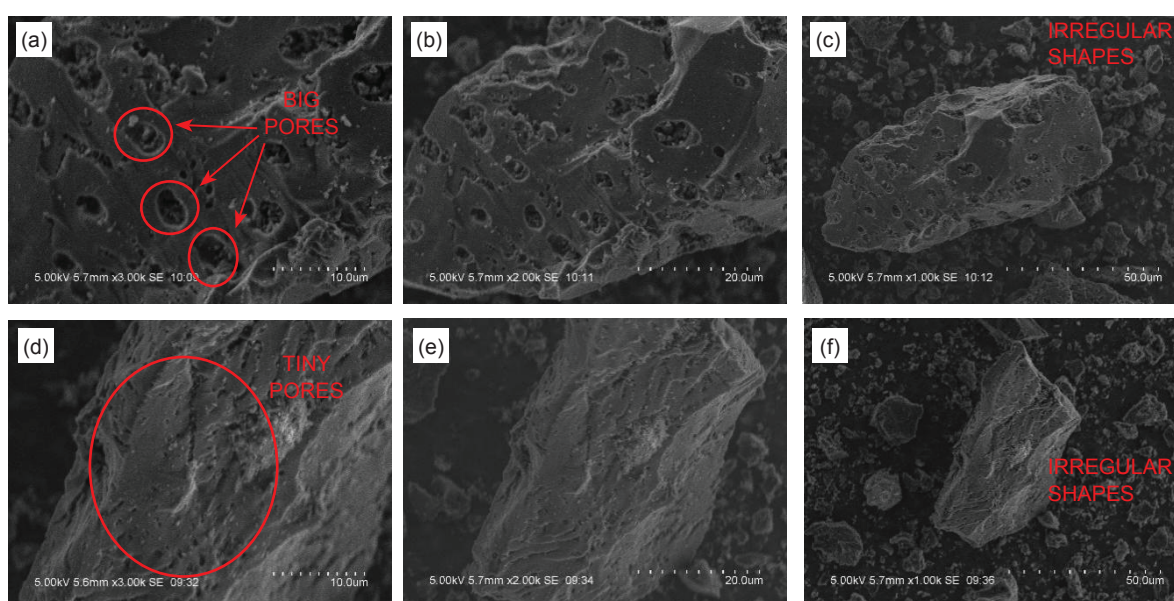


Figure 3. SEM images of PKSB at different magnifications (a) 1000 $\times$, (b) 2000 $\times$, (c) 3000 $\times$ and APKS at different magnifications (d) 1000 $\times$, (e) 2000 $\times$, (f) 3000 $\times$.

the rigidity of the material due to the extent of cross-linking and the reinforcement by fillers (Mohamad *et al.*, 2008; Sianturi and Surya, 2018). Both M_H and M_L of PKSB-filled NR composites increased as the PKSB loading increased as seen in Table 4. This may have been due to the increment of the PKSB content which increased the viscosity of the rubber composites and restricted the molecular motion of the macromolecules (Tomczyk *et al.*, 2020; Wang *et al.*, 2019). ΔM of formulation 1 showed the lowest value, which could be partly due to the absence of APKS and PKSB. Whereas, the ΔM of PKSB-filled NR composites showed an increasing trend as the filler loading increased which may be due to the formation of cross-linking between filler and rubber phases. Based on the SEM images of PKSB which displayed its porous structure, the pores provided sites for crosslink formation which caused ΔM of PKSB-filled NR composites to increase, thus improving the stiffness of the composites (Abdelsalam *et al.*, 2021; Kim *et al.*, 2020; Ngamsurat *et al.*, 2011). The incorporation of PKSB in the NR matrix did not affect the scorch time (t_{s2}) and the curing time (t_{90}). Meanwhile, the addition of APKS in the NR matrix did not change much on the M_H , M_L , ΔM , t_{s2} and t_{90} values as shown in Table 4. Hence, it could be said that the incorporation of APKS in NR composites regardless of any loading ratio was able to maintain the M_H , M_L , ΔM , t_{s2} and t_{90} values which would indicate appropriate processing safety, crosslink formation and cure time as there were only minor differences in curing characteristics between each formulation in APKS-filled NR composites (Sianturi and Surya, 2018). The torque values (M_H , M_L , ΔM) in APKS-filled NR composites showed minor differences which indicated that the filler loading between 5 phr to 50 phr had similarities in

terms of crosslinking sites that contributed to the filler-rubber matrix interactions. While t_{s2} and t_{90} for APKS also showed minor differences because the filler loading has similarity in terms of the heat distribution in the matrix due to the incorporation of filler (Mente and Motaung, 2016).

Swelling Properties

The degree of swelling (Q) and the rubber volume fraction (V_r) of the PKSB- and APKS-filled NR composites are shown in Tables 5 and 6. The absorption of toluene by rubber composites until the swelling equilibrium was achieved at room temperature was used to determine the Q values. The V_r value is the volume fraction of the equilibrium swollen rubber with filler, reflecting the rubber-filler interaction (Ismail and Shaari, 2010). Table 5 displays the decreasing trend of Q and the increasing trend of V_r for PKSB-filled NR composites. The Q value for formulation 1 was higher due to the absence of filler meanwhile PKSB-filled rubber composites showed a decreasing trend in swelling percentage, which could indicate high crosslink density, resulting in better rubber-filler adherence properties. The reduction of Q values also might be due to the mobility of polymer chains that restricted the penetration of toluene into the 3D network structure of composites. The increasing trend of V_r reflects the enhancement of PKSB-NR matrix attachments (Kim *et al.*, 2020; Nabil *et al.*, 2013). Meanwhile, the APKS-filled NR composites showed that the Q values fluctuated and the V_r values did not differ much as shown in Table 6. This occurred due to the formation of strong filler-filler interaction which influenced the immobilisation of rubber chains, thus giving better-swelling properties (Kim *et al.*, 2020).

TABLE 4. CURE CHARACTERISTIC OF PKSB AND APKS-FILLED NR COMPOSITES

No. of formulation	M_H (dN.m)	M_L (dN.m)	ΔM (dN.m)	t_{s2} (min)	t_{90} (min)
F1	11.62	0.58	11.04	1:17	2:30
F2	14.60	0.86	13.75	1:28	3:19
F3	15.86	0.75	15.11	1:10	2:36
F4	17.84	0.94	16.90	1:15	2:44
F5	20.16	1.43	18.73	1:26	3:14
F6	22.78	1.59	21.19	1:23	3:10
F7	23.05	1.39	21.67	1:25	3:17
F8	14.70	0.93	13.77	1:10	2:32
F9	15.05	1.17	13.88	1:09	2:22
F10	14.18	1.23	12.95	1:11	2:12
F11	14.33	1.64	12.69	1:39	3:21
F12	15.22	1.33	13.89	1:29	3:13
F13	13.19	2.20	10.99	2:05	4:45

TABLE 5. SWELLING PROPERTIES OF PKSB-FILLED NR COMPOSITES

No. of formulation	Q (%)	V _r
F1	363.10	0.15
F2	329.16	0.16
F3	310.00	0.17
F4	292.93	0.18
F5	288.57	0.18
F6	232.91	0.22
F7	225.53	0.22

TABLE 6. SWELLING PROPERTIES OF APKS FILLED NR COMPOSITES

No. of formulation	Q (%)	V _r
F8	315.19	0.17
F9	316.77	0.17
F10	329.84	0.16
F11	318.21	0.17
F12	305.46	0.17
F13	335.56	0.16

Tensile Properties

The tensile strengths of PKSB-filled NR composites are illustrated in *Figure 4a*, showing the increasing trend of tensile strength from 6 to 14 MPa as the filler loading increased. The porous structure of PKSB initiated better mechanical interlocking between PKSB and NR phases which could enhance

the stress transfer from the rubber matrix to filler particles, resulting in restricted mobility of polymer chains (Egwaikhide *et al.*, 2007; Lay *et al.*, 2020; Romli and Mamaud, 2017). Meanwhile, the tensile strength of APKS-filled NR showed the same trend as PKSB rubber composites at F8 (16 MPa) until F11 (18 MPa) as seen in *Figure 4b*. However, the tensile strength of APKS-filled NR composites was higher than PKSB-filled NR composites due to the wide porous surface area of APKS. The rough surface of APKS led to the enhancement of the rubber-filler adhesion via physical interaction. Up to F11, tensile strength started to deteriorate at high APKS loading which might be due to the aggregation of APKS particles. The aggregation of APKS particles led to producing weak points in the composites and hence decreased the tensile properties (Abdelsalam *et al.*, 2021).

Figures 4c and *4d* show the effect of PKSB and APKS loading on the elongation at break for NR composites. It could be seen that the increasing PKSB and APKS loadings decreased the elongation at break. The decreasing trend was caused by the presence of PKSB and APKS in the NR composites, restricting the mobility of NR chains and therefore causing composite failure at lower elongation. This also can be explained by the porous structure between PKSB and APKS in which the stress applied to filler-rubber matrix interfacial adhesion until at one time the stress was too much for a filler-rubber matrix that could cause the rupture to happen (Mente and Motaung, 2016).

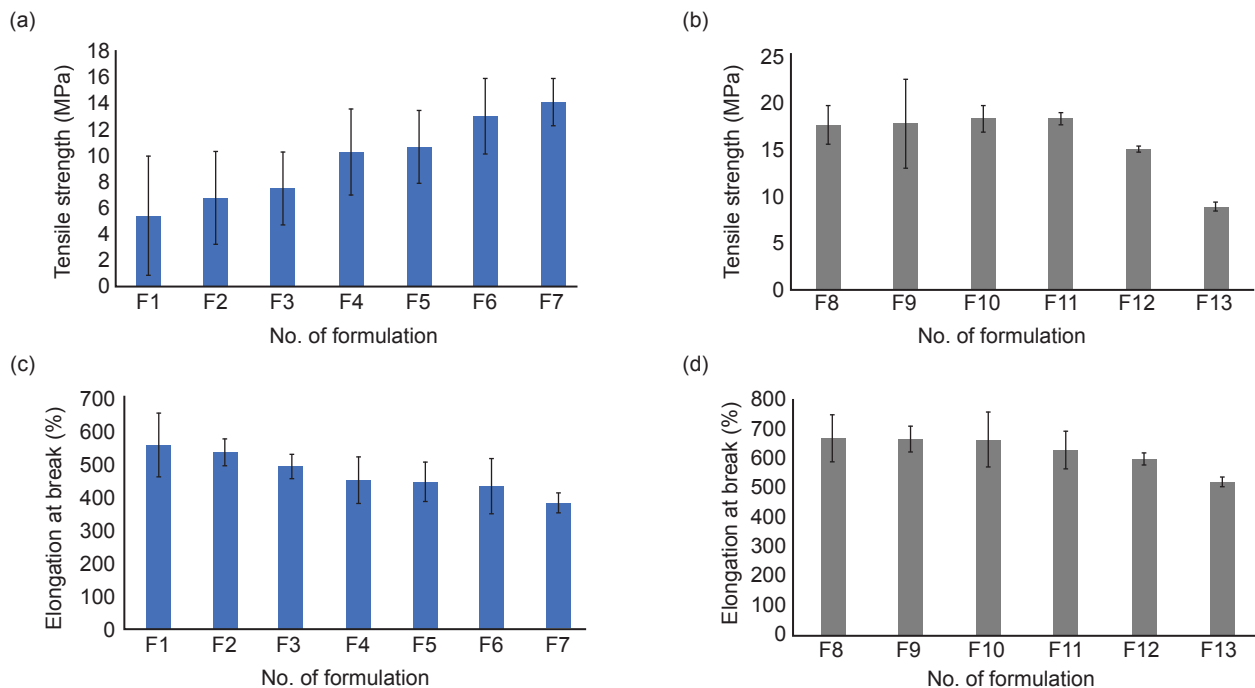


Figure 4. Tensile strength of rubber composites (a) PKSB-filled NR, (b) APKS-filled NR and elongation at break (c) PKSB-filled NR, (d) APKS-filled NR composites.

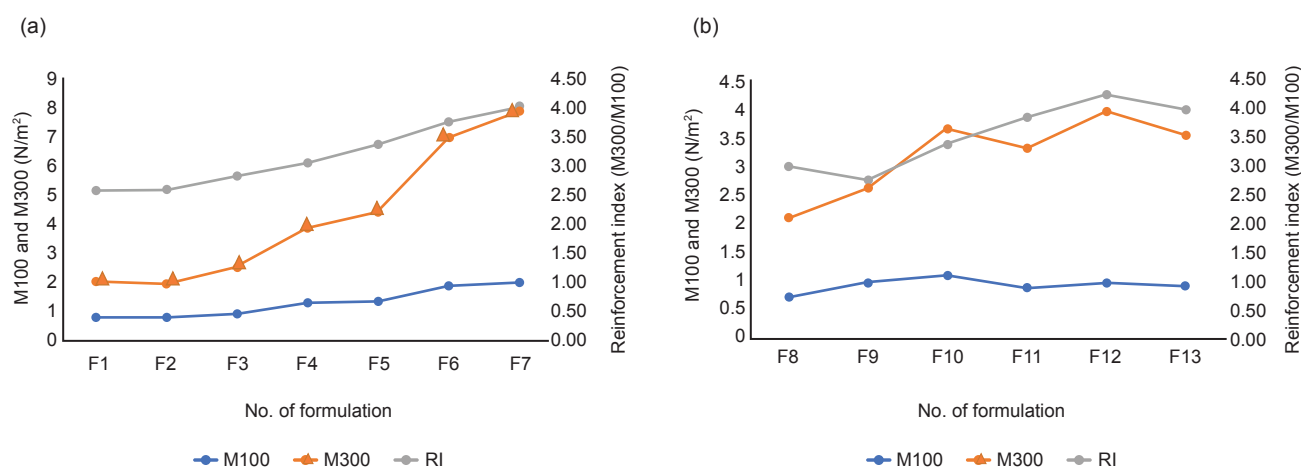


Figure 5. M100, M300 and RI. (a) PKSB-filled NR composites, and (b) APKS-filled NR composites.

The modulus at 100% (M100), 300% (M300) and the reinforcement index (RI) of PKSB- and APKS-filled NR composites are shown in Figures 5a and 5b. In both figures, M100, M300 and RI of PKSB- and APKS-filled NR composites increased as the filler loadings increased. This occurred due to filler-filler and rubber-filler interactions that contributed to the stiffness of composites (Ismail and Shaari, 2010; Lin *et al.*, 2016; Sobhy and Maziad, 2003). High filler content increased the probability of physical interaction between rubber, PKSB and APKS, leading to better reinforcement.

Comparative Study on PKSB, APKS and Other Biomasses

Table 7 displays the comparison in cure characteristics of PKSB and APKS with different types of biomass fillers used in rubber compounding which are coconut fibre, untreated kenaf and bamboo charcoal powder (BCP). PKSB recorded the highest maximum torque value compared to the other renewable fillers because the macromolecular rubber chain mobility decreased as the filler

loading increased, resulting in more rigid rubber composites. The M_H values of APKS, coconut fibre and kenaf were slightly lower due to the particle size and the surface area of the biomass fillers, which may affect the results obtained (Egwaikhide *et al.*, 2007; Ismail *et al.*, 2010). However, untreated kenaf and BCP showed longer curing times (t_{90}) compared to the other fillers. This may be due to the low viscosity of the fibres, hence low energy was needed to enhance the adhesion between the filler and rubber matrix (Ibrahim *et al.*, 2018; Ismail *et al.*, 2010).

Table 8 shows the comparison of PKSB, APKS and other biomass fillers in terms of swelling percentage, Q . PKSB showed a slightly lower swelling percentage, while APKS and coconut fibre had the highest Q value, compared to bamboo charcoal due to their large surface area and porous structure. A previous study stated that, as the filler loading in the rubber matrix increased, more impediments were formed for the diffusing molecule, reducing the amount of solvent penetrated (Egwaikhide *et al.*, 2007).

TABLE 7. COMPARISON OF PKSB AND APKS WITH OTHER RENEWABLE FILLER IN CURE CHARACTERISTICS

Types of filler	M_H (dN.m)	t_{s2} (min)	t_{90} (min)	References
PKSB	11.62-23.05	1:10-1:28	2:30-3:19	-
APKS	14.70-15.22	1:09-2:05	2:12-4:45	-
Coconut fibre	9.48	0:30-0:40	0:40-1:00	Egwaikhide <i>et al.</i> (2007)
Untreated kenaf	7.00-22.00	2:00-4:50	3:90-6:80	Ismail <i>et al.</i> (2010)
BCP	-	1:20-1:30	5:00-6:00	Ibrahim <i>et al.</i> (2018)

TABLE 8. COMPARISON OF PKSB AND APKS WITH OTHER RENEWABLE FILLERS IN SWELLING PROPERTIES

Types of filler	Swelling percentage, Q (%)	References
PKSB	226-329	-
APKS	305-336	-
Coconut fibre	262-398	Egwaikhide <i>et al.</i> (2007)
Bamboo charcoal	270-320	Chen <i>et al.</i> (2019)

The comparison between PKSB, APKS, coconut fibre, bamboo charcoal and untreated kenaf is shown in Table 9. APKS-filled NR composites had higher tensile strength and elongation at break compared to other fillers. This is because APKS possesses a higher carbon content that could provide a greater reinforcement effect between the filler and rubber matrix. The tensile strength and the elongation at break of other renewable fillers are lower due to their nature and the inability of the filler to adhere as well as to support the stress transferred from the polymer matrix (Egwaikhide *et al.*, 2007). For M100, the results obtained for every filler were almost similar and this might have contributed to the stiffness of the rubber composites.

TABLE 9. COMPARISON OF PKSB AND APKS WITH OTHER RENEWABLE FILLERS IN TENSILE PROPERTIES

Types of filler	Tensile strength (MPa)	Elongation at break (%)	M100	References
PKSB	5-14	385-561	0.74-1.97	-
APKS	8-18	519-669	0.70-1.08	-
Coconut fibre	1-6	300-850	1.00-2.00	Egwaikhide <i>et al.</i> (2007)
Bamboo charcoal	6-8	450-600	0.50-1.20	Ibrahim <i>et al.</i> (2018)
Untreated kenaf	4-21	210-1 100	0.90-4.00	Ismail <i>et al.</i> (2010)

CONCLUSION

The overall findings of PKSB and APKS as potential bio-filler compared with other biomass such as coconut fibre, kenaf and BCP were able to be investigated. The SEM images showed APKS-filled NR composites produced a well-developed porous structure with a rough surface area than PKSB-filled rubber composites, which could improve the mechanical interlocking of rubber-filler, providing better mechanical properties for the rubber composites. PKSB-filled rubber composites had higher torque values compared to other biomass fillers like coconut fibre, kenaf and BCP

in terms of curing properties, and could provide more rigid and stiff composites. However, APKS-filled NR composites had better processability than PKSB since t_{s2} and t_{90} were not affected by the filler loadings. PKSB had slightly better-swelling percentages compared to APKS and other fillers as the filler loadings increased, which indicated better rubber-filler interaction and the formation of a crosslinked network between rubber and filler bases. For mechanical properties, APKS possessed higher tensile strength and elongation at break with lower modulus compared to other fillers. This shows good interfacial adhesion between the rubber and the PKS filler. The optimum loading for APKS-filled NR composites is at 40 phr. Therefore, APKS has a high potential to be used as a semi-reinforcing bio-filler in the rubber industry due to its enhanced properties in comparison with other biomass fillers.

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PERFORMANCE EVALUATION OF A TIPPING DEVICE FOR FRESH FRUIT BUNCH (FFB) LOADING

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ABSTRACT

Manual loading of oil palm fresh fruit bunches (FFB) involves picking up the bunches with an iron spike and dropping them into a bin or tractor-trailer. Workers need to repetitively lift a load above their shoulders hence, giving extreme strain to the muscle. To overcome this issue, a prototype was designed and developed to directly transfer FFB. The device is equipped with a hydraulic system and powered by a battery. There is a need to assess the overall performance of the device compared with the manual. The time taken to transfer a different number of bunches, time taken for total FFB evacuation, productivity and battery depletion rate was evaluated. Three-wheel transporters were used and a plot with medium-size FFB was selected. The study indicated that the device was able to handle 400 kg of loads in 38.1 s. It was found that by using the device, unloading activity will only consume about 4% of the total FFB collection time. The study showed that the machine helped the workers by reducing their time to complete the task by 35% more than the manual. In conclusion, the device functioned and performed as it is designed. Workers can now directly transfer the FFB into a bin at a reduced time.

Keywords: fresh fruit bunch, hydraulic powerpack, lifting device, oil palm, small transporter.

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INTRODUCTION

The economic performance of the Malaysian palm oil industry was very impressive with the value of palm oil exports of RM64.6 billion despite the Covid-19 pandemic situation in 2021 (Parveez *et al.*, 2022). With a total oil palm planted area of 5.74 million hectares in 2021, the industry is facing difficulties to produce their optimum output due to the labour shortage, low level of mechanisation and escalating cost of fresh fruit bunches (FFB) production. The oil palm industry is very much dependent on labour. The lack of mechanisation options in plantations creates an over-reliance on manual labour for all key activities, especially harvesting.

The introduction of mini-tractors with trailers in the early 80s for in-field FFB collection was welcomed

by the industries. Besides improving productivity and earnings, the mini-tractors also improved overall harvesting standards (Yaakob and Toh, 1988). The present manual loading system involves picking up the bunches with an iron spike and dropping them into a trailer that is pulled by a mini-tractor. Besides tractor-trailers, the use of 3 to 5 t lorries is also common in the industry. The average output of a mini-tractor per day is about 20 t FFB. The introduction of a mechanical loader (Grabber) in 1992 further improved the method for FFB collection (Ahmad and Ahmad, 1992). Among factors that were identified as critically influencing the efficiency of FFB evacuation process are the skills of the driver, terrain conditions, labour availability and harvesting technique (Mohd Lutfi and Hairul Rizad, 2020).

The introduction of a bin system in the oil palm plantations thereafter has given significant impacts on more efficient FFB collection and evacuation. Many plantations adopted this system and most of them integrated it with Mini-tractor Grabber with a high lift trailer. However, not all plantations are

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ready to accept this technology due to terrain and budget constrain, especially among field contractors and smallholders.

Besides mini-tractor, there are several small FFB transporters available for use in oil palm plantations. These machines are widely used and usually drop their loads on the ground at the collection point and the FFB will then be manually transferred into the bin or lorry (Azali *et al.*, 2015; Che Ahmad *et al.*, 2022). This multi handling activities also promote lower oil quality as damaged FFB will produce a higher percentage of free fatty acid (FFA) (Turner and Gillbanks, 2003).

Furthermore, these small FFB transporters are unable to be fully integrated with the bin system due to the limited tipping height. The average bin height is around 1.2 m to 1.8 m depending on the bin's size and this is far beyond the normal tipping height of small FFB transporters (about 0.3 m to 0.5 m). Though there are options such as the small FFB transporters to be fitted with a scissor lift or high pivot discharge mechanism to enable them to unload the FFB into the bin, these options are less favourable as they incur extra cost.

There are some cases where the use of a shallow bin is required when a small type of transporter is used. However, the load capacity is limited when this type of bin is in use. Another option is by using a bin with a door or gate system where the infield transporter can move to enter the bin to unload the FFB. The only drawback is that manual loading is still required when the bin is almost full as the transporter cannot move into the bin.

From ergonomic and bio-kinetic aspects, the current manual loading activities impose a risk of injury to the workers. Workers need to repetitively lift a load above their shoulders hence, giving extreme strain on the muscles and joints (Ng *et al.*, 2013; Nur Syazwani *et al.*, 2016). A large number of bunches will exhaust the workers by the end of the day. A solution is thus, needed to reduce these risks.

MATERIALS AND METHODS

Prototype Design and Development

Key considerations in the design process to develop the device were:

- Mobile-type device for ease of mobility in the field
- Able to lift and transfer the FFB into a bin, lorry or tractor-trailer (approximately 1.8 m height)
- Can be attached to existing small transporter with minor modifications
- Simple hydraulic lifting mechanism that could lift 400 kg loads (FFB)
- Independent power source to run the system

The computer-aided-design (CAD) model of the device was done using the Autodesk Inventor software.

Time Taken to Complete One Operation Cycle with Different Number of Bunches

A study was carried out over six sets (5, 10, 15, 20, 25 and 30 bunches per load). The total weight for each set was recorded using a weighing bridge at the collection centre. Each set of bunches was placed in the device's bucket and times were recorded once the start button was pushed until all the FFB were transferred into the bin and the bucket frame was back to its standby position. Three replications per set were performed and the average value was recorded.

Duration for Total Operation

Using a three-wheel transporter with one driver and one helper, the duration for activities involving the use of the device was recorded, *i.e.*, the travelling time of the vehicle to search and collect FFB from the field and transport to the bin, time taken to unload FFB from vehicle to the tipping device and time taken for the tipping device to unload FFB into the bin and back to its standby position. Six replications were conducted and the average value was recorded.

Performance Comparison

Two units of three-wheel transporters were used to collect and transport the FFB from the field to the bin (one driver and one helper for each machine). One transporter was tasked to unload the FFB manually into the bin, while another one was tasked to unload the FFB using the device. The number of bunches and time taken to complete the overall loading task for both methods were recorded for 5 replicates. Medium-size FFB (12-15 kg per bunch) were selected from the same plot.

One-way analysis of variance (ANOVA) was used to determine the significant differences between the average time taken between the machine and the manual method. The value of the degree of freedom (DOF), F-statistic, and *P*-value were calculated. To determine the significant responses, values of F-statistic must be higher than F-critical in the distribution table, which is 3.55 at a 95% confidence interval. The F-critical value can be defined by referring to the *f* distribution table at a 95% of confidence level, *i.e.*, $F_{0.05}(f_1, f_2)$. The numerator value, f_1 is the degree of freedom of response, and the denominator, f_2 is the deviation of DOF. F-statistic can be calculated using Equation (1):

$$F_{\text{statistic}} = \frac{\text{Mean square, MS (between response)}}{\text{Mean square, MS (within response)}} \quad (1)$$

Battery Voltage Depletion

Depletion was calculated from the differences in voltage before and after each operation. The voltage of each battery was recorded before and after each operation using a digital multi-meter (20 replicates). The average load for each tipping operation was approximately 400 kg.

Operational Cost Analysis

A simple operational cost analysis was calculated using the straight-line depreciation formula.

RESULTS AND DISCUSSION

Prototype Design and Development

The body consists of two major components, *i.e.*, the mainframe and bucket frame. The mainframe was made from a combination of rectangular mild steel and designed to accommodate a bucket frame, hydraulic power pack, hydraulic cylinders, power source, tyres and towing system (*Figure 1*). The bucket frame was made from a combination of rectangular, plate and rod mild steel. It was designed for the temporary storage of about 400 kg FFB. The bucket frame was attached to two cylinders so that it can be pushed to 130° from the pivot points. This prototype was designed to service bins of up to 1.8 m in height. The hydraulic power pack system was powered by two units of 12 V lead-acid batteries that were connected in series. The technical specifications of the device are shown in *Table 1*.

Proposed Working System (Three-wheeler transporter and bin system)

The tipping device will be attached to a transporter that will move to the bins in the field.

TABLE 1. TECHNICAL SPECIFICATIONS OF A TIPPING DEVICE FOR FRESH FRUIT BUNCH (FFB) LOADING

Dimension, L x H x W (mm)	1980 x 1828 x 1370
Hydraulic power pack	Model: D222XXI DC 24V, 2.2kW 2600 RPM, 6.0 Nm
Working height (m)	1.8
Tyre size	5.00 -010 NHS 12 P.R
Battery	2 x 12V 1000 mAh

These bins were placed strategically at points that were determined earlier. Thereafter, the device will be detached and placed at the side of the bin.

The operator/driver then starts his routine by collecting FFB in the field. Once fully loaded, the transporter will then manoeuvre to the bin to unload the FFB. The transporter will then unload the FFB into the bucket of the device in reverse position. Once completed, the transporter will then move forward (about 1-2 m from the device) and the device will lift the bucket to transfer the FFB into the bin (*Figure 2*) with a single press of a switch. The device will automatically retract to its original position once empty. The transporter will then immediately move back to the field, looking for more bunches without the need to wait and load the FFB manually.

Time Taken to Complete One Operation Cycle with Different Number of Bunches

A study showed that the prototype could lift up to 400 kg bunches in 38 s. The average time taken to complete one operation cycle was about 34-38 s depending on the number of loads (*Figure 3*). Longer time is needed to lift higher loads. This situation is normal due to the inability of the hydraulic motor/ pump to deliver sufficient flow to the hydraulic cylinders, especially when a battery is used as a power source for the hydraulic power pack.

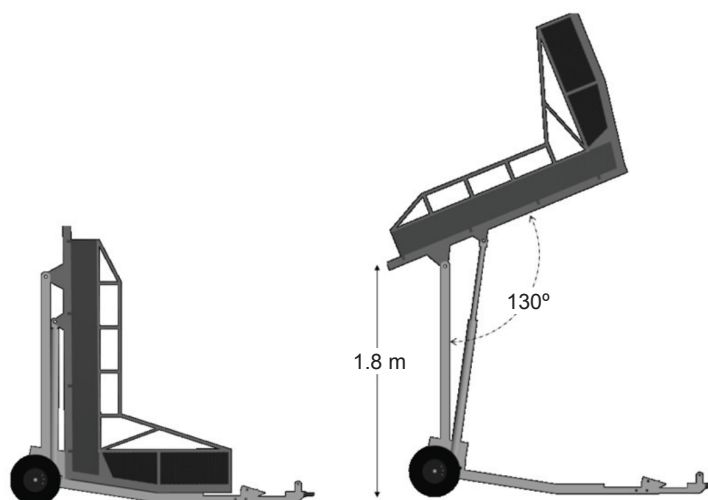


Figure 1. Side views of the device during standby and tipping positions.



Figure 2. Prototype in tipping position during a field test.

Duration for Total Operation

The average time taken to complete the total operation was about 315 s or 5.24 min (Table 2). By using the device, the FFB unloading activity only consumed about 4% of the total operation time. It was found that 88% of total operation time was consumed for travelling and searching for FFB. This is almost similar to the findings reported by Azmi (2000) and Turner and Gillbanks (2003), where the duration for travelling and picking FFB was around 77%-80% of the collection time. Hence, improvement through machinery enhancement and a better working system is thus necessary to speed up the FFB collection process.

Performance Comparison

A comparison study was conducted to compare the performance of the machine and manual loading. It was found that the average time taken for the

TABLE 2. SUMMARY OF DURATION FOR TOTAL OPERATION

Activities	Time (s)	Percentage (%)
Travelling time for in-field transporter to search and collect FFB from field to bin	276.6 ± 24	87.9
Time to unload FFB from the machine to the tipping device	26.2 ± 1.1	8.3
Time for the tipping device to unload FFB into the bin and back to a ready position	11.9 ± 0.8	3.8
Total	314.7	100.0

machine to complete the loading task was shorter than manual loading. The machine performed better than the manual method as analysed by ANOVA through the calculation of time needed to complete the loading tasks (Table 3). F-critical was $F_{0.05}(1,8) = 5.318$ at a 95% confidence level ($\alpha = 0.05$). The F-statistic for the time responses is greater than F-critical with P -value lower than $\alpha = 0.05$. Thus, there is a significant difference between the means of time responses for both methods indicating that the machine indeed helped the operators by reducing their time to complete the task (35% faster).

A worker needs to repetitively lift the FFB above his shoulders with an iron spike and drop it into a 1.8 m height trailer in the manual method as opposed to the machine where the worker is required to only press the push button and the FFBs will be automatically transferred into the trailer. This is the biggest advantage of the machine besides consuming less energy to complete the task. However, it was found that a minimum loading activity was still involved in the process, especially when arranging FFB at the top layer of the bin. This arrangement of FFB is needed to maximise carrying the load and also to provide additional safety during transportation. It was also found that the total number of FFB that could be handled by using the machine was higher than in the manual method, 356 bunches and 320 bunches respectively.

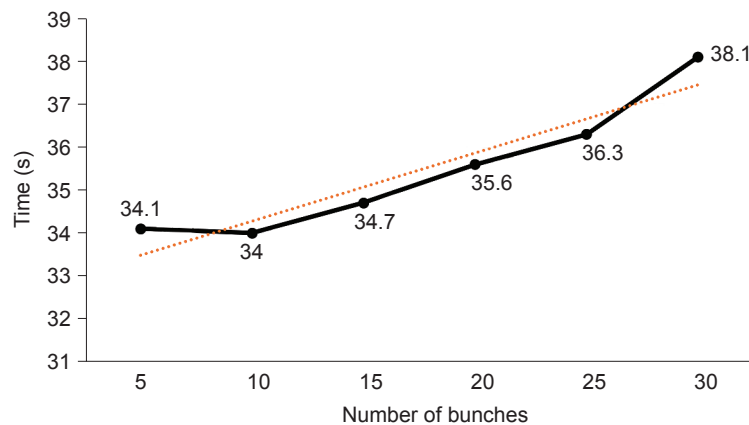


Figure 3. Time taken to complete one operation cycle with a different number of bunches.

TABLE 3. ONE-WAY ANOVA ANALYSIS OF TIME NEEDED TO COMPLETE THE LOADING TASK USING MACHINE AND MANUAL METHOD

Groups	Count	Sum	Average	Variance
Machine	5	1 710	342	1 252.5
Manual	5	2 310	462	256.5

Source of variation	SS	df	MS	F-statistic	P-value	F-critical
Between groups	36 000	1	36 000.0	47.71371769	0.000124	5.317655
Within groups	6 036	8	754.5			

Note: *F-statistic > F-critical = significant difference.

Battery Voltage Depletion

The device's battery depletion was about 0.02 ± 0.033 V per operation (Table 4). It was estimated that the battery can last up to two days of operation before it needs to be charged. However, this estimation varies depending on a daily usage, charging procedure, age and maintenance of the batteries.

TABLE 4. BATTERY DEPLETION PER OPERATION

Description	Voltage (v)
Battery depletion/operation (v)	0.02 ± 0.033

Operational Cost Analysis

Based on the estimated machine price of RM12 000, the economic life of 5 years, productivity of 25 t day^{-1} , labour cost of $\text{RM}45 \text{ day}^{-1}$, 26 days working day a month and using the straight-line depreciation formula, the cost of using the machine is around $\text{RM}2.26 \text{ t}^{-1}$ FFB. With the expected labour saving of at least one person, the operational cost of the machine is thus justified.

CONCLUSION

A lifting device to assist FFB loading was developed and evaluated in the field. The concept of a mobile-type device using a simple hydraulic tipping mechanism is proven to be effective to accelerate the FFB loading operation. With the assistance of this device, workers can directly transfer the FFBs into a bin at a reduced time without exerting their energy excessively as only minimum manual loading activities were involved. It is envisaged that the technology could provide numerous advantages towards reducing labour requirements, providing better working conditions, and reducing multiple handling operations which in the end will lead to higher productivity.

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DEVELOPMENT AND IN-HOUSE VALIDATION OF MULTI-RESIDUE METHOD FOR TRIAZOLE FUNGICIDES IN PALM KERNEL, MESOCARP AND RAW AGRICULTURE COMMODITY (RAC) USING GAS CHROMATOGRAPHY-MICRO ELECTRON CAPTURE DETECTOR (GC- μ ECD)

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ABSTRACT

A simple, fast and rapid multi-residue method for the analysis of triazole fungicide residues in palm kernel, mesocarp and raw agricultural commodity (RAC) was developed and validated using gas chromatography-micro electron capture detector (GC- μ ECD). The method was developed from a modified QuEChERS extraction method and followed by dispersive solid-phase extraction (d-SPE) clean-up procedure. Five triazole compounds were studied, namely tetraconazole, penconazole, hexaconazole, propiconazole and epoxiconazole. Optimisation of different solvent extractions was conducted in order to find a suitable solvents for triazole residue extraction. The developed method was validated in-house and satisfied the important validation parameters such as recovery, repeatability and reproducibility based on SANTE guideline. The recoveries for all fungicides at fortification levels of 0.1, 0.5 and 1.0 mg kg⁻¹ in palm kernel, mesocarp and RAC were in the range of 80% to 108% with relative standard deviations (%RSD) of less than 10%. The limit of detection (LOD) and limit of quantification (LOQ) for the developed method were estimated to range between 0.12 and 0.23 mg kg⁻¹ and 0.31 and 0.7 mg kg⁻¹, respectively.

Keywords: mesocarp, modified QuEChERS, palm kernel, raw agricultural commodity (RAC), triazole.

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INTRODUCTION

Palm oil is one of the most versatile and efficient vegetable oils traded worldwide. Like any other major vegetable oil, palm oil trading is subjected to many international trading specifications and regulations. CODEX Standard 210-1999 has defined palm oil and palm kernel oil as products derived from fleshy mesocarp and kernel of the fruit of the oil palm (*Elaeis guineensis*), respectively (CODEX, 1999). CODEX classified oil palm fruit as Processed

Food of Plant Origin under Nuts and Seeds (SO) and Group 023, Oilseed (FAO, 2021). Oilseed consists of seeds from a variety of plants used in the production of vegetable oils, seed meals and cakes for animal feed.

CODEX report on pesticide residues in food stated that any processed foods obtained from primary food commodities, which it is derived, needs to be analysed separately (CODEX, 1993). If the residue higher in the processed food than the RAC, separate maximum residue limit (MRL) should be considered for the processed food. Crude palm oil (CPO) and crude palm kernel oil (CPKO) are categorised under Class D: Processed Food of Plant Origin and divided into Type 067: Vegetable oil, crude which is derived products of plant

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origin. Based on this classification, oil palm fruits are considered as RAC and therefore the whole commodity components need to be analysed. According to the CODEX food category system, RAC means any food in its natural state intended for processing into food for sale to the consumer or as a food without further processing. It includes irradiated primary food commodities and products after removal of certain parts of the plant or parts of animal tissue (FAO, 2009). However, there is no published method available for the determination of pesticide residues in oil palm fruits and RAC.

On top of that, Codex Alimentarius Commission (CAC) also emphasised on other parts of commodities which are used as animal feeds. Palm kernel cake (PKC) is mainly used as a common ingredient for animal feed formulation. These commodities are listed under Class C: Primary Animal Feed Commodities. Previous studies had shown that PKC has high nutritional values (protein, fibre or lipids), provides balanced diet in amino acids and metabolisable energy (Alimon, 2004; Sharmila *et al.*, 2014; Sundu *et al.*, 2006). Many studies have reported that fermented PKC has some probiotic activity capable to enhance the performance of broilers and improve product quality (Aladi *et al.*, 2017; Halim *et al.*, 2021; Hanafiah *et al.*, 2017; Kalidas *et al.*, 2017). Despite the widespread uses of palm kernel in animal feed formulations, analytical method for the determination of pesticide residues in palm kernel matrices has never been established. There are many published methods for the determination of pesticide residues in CPO and CPKO (Halim *et al.*, 2020; Khairuddin *et al.*, 2021; Muhamad *et al.*, 2008; 2012a; Sobhanzadeh and Nemati, 2012; Sulaiman *et al.*, 2022; Yeoh *et al.*, 2006; Zainudin *et al.*, 2009). Under Malaysian Palm Oil Board Code of Practices (MPOB CoP), clause 4.8.2 suggests that CPO for edible purposes should be monitored frequently for pesticide residues (MPOB, 2021). However, to date, analytical method for the determination of pesticide in mesocarp is not available despite mesocarp is the source for CPO.

QuEChERS, is a common method used for multi-residue pesticides analysis in fruits and vegetables. QuEChERS stands for Quick, Easy, Cheap, Effective, Rugged, and Safe, is a simple sample preparation method that was introduced by Anastassiades *et al.* (2003). The principle of this method is based on a salt-out extraction using solvents; commonly acetonitrile followed by a d-SPE clean-up procedure using sorbents such as magnesium sulphate (MgSO_4) and primary secondary amine (PSA). QuEChERS method provides reliable results, simple analytical process for extraction and clean-up steps, less reagents consumption as well as minimum utilisation of laboratory instrument and apparatus.

Nonetheless, further improvement to this method will broaden its applicability to other wide variety of commodities. This method gives a huge advantage to the food safety industry as it is cost-effective.

The most frequent and widely used sorbents in QuEChERS method were PSA, graphite carbon black (GCB) and octadecyl (C18) sorbents combination and anhydrous MgSO_4 for clean-up step (Anagnostopoulos and Miliadis, 2013; Deme *et al.*, 2014). Numerous published reports on the analysis of pesticide residues in vegetable oil using d-SPE with different sorbent combinations and anhydrous MgSO_4 for clean-up step. PSA is a well-known sorbent for removing of fat content when necessary, the chemical structure of PSA provides high retention of free fatty acids and other polar matrix compounds (Deme *et al.*, 2014). GCB has a strong affinity for planar molecules, and thus effectively removes pigments such as chlorophyll and carotenoids, as well as sterols present in foods (Anastassiades *et al.*, 2003). Meanwhile, C18 can act as a clean-up sorbent resulted in a chromatogram with lower background and interfering peaks (Sobhanzadeh *et al.*, 2012). However, the use of PSA and GCB may raise some concerns especially on the recovery of pesticides with planar structures *e.g.*, chlorothalonil and thiabendazole (Lentza-Rizos *et al.*, 2001; Muhamad *et al.*, 2012).

Many studies reported the performance of modified QuEChERS method in analysing pesticide residues in various matrices such as edible oils (Chamkasem and Harmon, 2015; Halim *et al.*, 2020; Theurillat *et al.*, 2021), fruits and vegetables (Dong *et al.*, 2019; Li *et al.*, 2017) and soil (Asensio-Ramos *et al.*, 2010; Caldas *et al.*, 2011). Slight method modification involving the inclusion of liquid-liquid partition step and additional of water and iso-octane to the acetonitrile extracts facilitate the separation of the oil layers (Marchis *et al.*, 2012). New adsorbent materials such as cetyltrimethylammonium bromide (CTAB)-modified zeolite, synthetic magnesium silicate (florisil) and molecularly imprinted polymer (MIP) were also used as clean-up materials after the QuEChERS extraction (Batlokwa *et al.*, 2013; Salisaeng *et al.*, 2016; Wang *et al.*, 2017). Recently, metal-organic frameworks (MOFs), UiO-66 has shown its effectiveness as adsorbent in d-SPE clean-up for eliminating matrix interference compounds for determination of OP pesticides in edible vegetable oils (Mao *et al.*, 2019).

Fatty food commodities such as olives, oil seeds, oils, nuts, milk and other dairy products, fish and meat require a slight modification of the QuEChERS method. Specific modifications of QuEChERS method are crucial in order to obtain good recovery values and satisfactory extract purification (Rejczak and Tuzimski, 2015). Fatty foods pose great challenge due to some of the lipids are coextracted with acetonitrile and may cause difficulties in subsequent

analysis or some fat-soluble nonpolar analytes could persist in fatty food samples and give poor extraction efficiency rates (Muhamad *et al.*, 2012a). The aim of this study is to develop fast, simple and reliable multi-residue method for determination of triazole fungicide in palm kernel, mesocarp, and RAC. Quantification of triazole fungicide residues was performed using GC- μ ECD.

MATERIALS AND METHODS

Chemicals and Reagents

Methanol and acetonitrile of pesticide grade were purchased from Merck (Darmstadt, Germany). Formic acid and acetic acid for mass spectrometry with purity of 98.0% were obtained from Sigma-Aldrich (St. Louis, USA). Sodium chloride (NaCl) and magnesium sulphate (MgSO_4) were purchased from Sigma-Aldrich. For the d-SPE adsorbents, bulk PSA, DSC-18 (C18) and ENVI-Carb GCB were obtained from Supelco Inc. (Bellefonte, PA, USA). Pesticide reference standard of triazole group was purchased from Dr. Ehrenstorfer (Augsburn, Germany) with purity of 98.8%.

Instrumentation

Gas chromatography-micro-electron capture detector (GC- μ ECD) (Agilent Technologies 7693 Series) equipped with an automated injector was used throughout this study. The system was operated with an HP-5 (5% phenylmethylsiloxane) capillary column (30 m long, 250 μm internal diameter and 0.25 μm thickness). The column oven temperature was first set at an initial temperature of 55°C and maintained for 1 min. The temperature was then increased to 220°C at 5 min and kept at the final temperature of 300°C for 8 min. The injector in spitless mode and detector temperature were set at 250°C and 280°C, respectively. Nitrogen was used as

carrier and make-up gas, with a flow rate of 0.8 and 60 mL min^{-1} , respectively. The injection volume was set at 1.0 μL .

Preparation of Reference Solution

Stock solution at concentration of 1000 mg L^{-1} of individual triazole fungicide was prepared by dissolving the appropriate amounts of individual triazole compound in acetonitrile into 10 mL volumetric flask. Then, the mixed reference stock solution (100 mg L^{-1}) containing all the triazole analytes was prepared by pipetting 1 mL of each individual stock solutions into a 10 mL volumetric flask and adding acetonitrile before mixing the solution for equilibrium. An intermediate working reference solution was prepared at the concentration of 10 mg L^{-1} through dilution using acetonitrile solvent in 10 mL volumetric flask. All solutions were stored in the dark at 4°C prior to analysis.

Palm Kernel, Mesocarp and RAC Samples for Fortification

Fresh mesocarp and palm nuts were collected after manual pressing of oil palm fruit in laboratory. Palm nuts sample was washed with hexane to remove oil, air-dried and manually crush to separate shell and kernel. Then, palm kernel was ground to powder form using laboratory blender (Waring 8010S/G). Fresh mesocarp was air-dried, cut into size of 0.5-1.0 cm using scissors and ground to homogeneous size. Fresh RAC was cut layer by layer into small pieces (0.2-0.5 cm) and ground using laboratory blender. All samples (palm kernel, mesocarp and RAC) were used for blanks and fortified samples in validation and matrix-matched standards preparation for calibration. The samples were stored in the freezer (-4°C) prior to analysis. Processed palm kernel, mesocarp and RAC samples are shown in *Figure 1*.

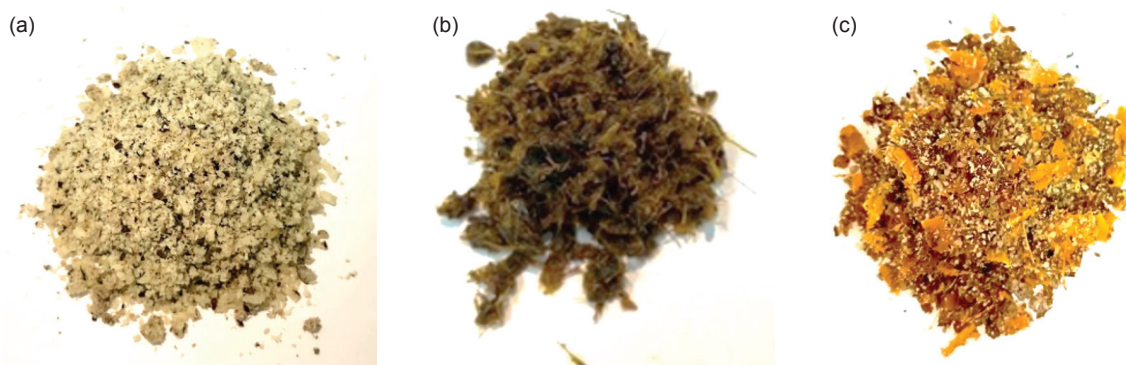


Figure 1. Processed (a) palm kernel, (b) mesocarp and (c) RAC samples.

Establishment of Matrix-Matched Calibration Curve

Matrix-matched calibration was established in a series of mixed standard at concentration of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mg L⁻¹ in palm kernel, mesocarp and RAC blank extracts. Samples of 1.0 g palm kernel, mesocarp and RAC were weighed in 50 mL screw cap centrifuge tubes and fortified with working standard solution to give final concentrations of 0.5, 1.0 and 5.0 mg g⁻¹. The tubes were mixed well using a vortex mixer and allowed to stand for 10 min to reach equilibrium. Then, 10 mL of acidified acetonitrile (1% acetic acid) was added into the spiked samples followed by 4 g of MgSO₄ and 1 g of NaCl. The tubes were then shaken for 3 min using a vortex mixer and underwent centrifugation at 5000 rpm for 5 min. Thereafter, 1 mL of supernatant was transferred into a d-SPE tube containing various amounts of MgSO₄, PSA and GCB. The matrix effect was calculated and expressed as percentage difference of the slope of the calibration curve in solvent and in matrix using Equation (Ferrer *et al.*, 2011):

$$\% \text{ Matrix effect (ME)} = \left[\frac{\text{slope matrix}}{\text{slope solvent}} - 1 \right] \times 100\% \quad (1)$$

Modified QuEChERS Method

Preliminary tests were conducted to evaluate the modified QuEChERS with original QuEChERS (Anastassiades *et al.*, 2003). For the evaluation of the extraction step, blank matrix was spiked at 1 mg kg⁻¹

in five replicates and matrix match calibration was used for recovery calculation.

One gram of sample was weighed in a 50 mL polypropylene (PP) tube. The extraction solvent was added and the samples were hand shaken for 1 min. The solvent added in each test tube was as follows: (a) 10 mL of acetonitrile (100%), (b) 10 mL of 1% (v/v) acetic acid in acetonitrile, (c) 10 mL of 3% (v/v) acetic acid in acetonitrile, (d) 10 mL of 1% (v/v) formic acid in acetonitrile and (e) 10 mL of 3% (v/v) formic acid in acetonitrile. Then, 4 g of MgSO₄ and 1 g of NaCl were added to initiate partitioning process. The centrifuge tube was vortexed for 3 min for palm kernel and RAC and 5 min for mesocarp, followed by centrifugation at 5000 rpm for 5 min. Afterwards, 1 mL of supernatant was subjected to clean-up step using d-SPE. The optimum extraction solvent was selected upon the highest value of mean recovery of the analytes. The flow chart of QuEChERS procedure for the pesticide residual analysis is summarised in Figure 2.

Dispersive Solid-Phase Extraction (d-SPE) Clean-up

Various sorbent combinations were investigated using d-SPE for clean-up procedure prior to GC- μ ECD analysis. An aliquot (1 mL) of the upper layer was transferred into a d-SPE tube containing one of the following for testing: (1) 0.45 g of anhydrous MgSO₄ and 0.20 g PSA, (2) 0.45 g of anhydrous MgSO₄, 0.25 g of PSA and 20 g of GCB, (3) 0.45 g of anhydrous MgSO₄, 0.25 g of PSA and 25 g of GCB. The tubes were then tightly capped and shaken for 1 min before centrifugation at 3000 rpm for 1 min.

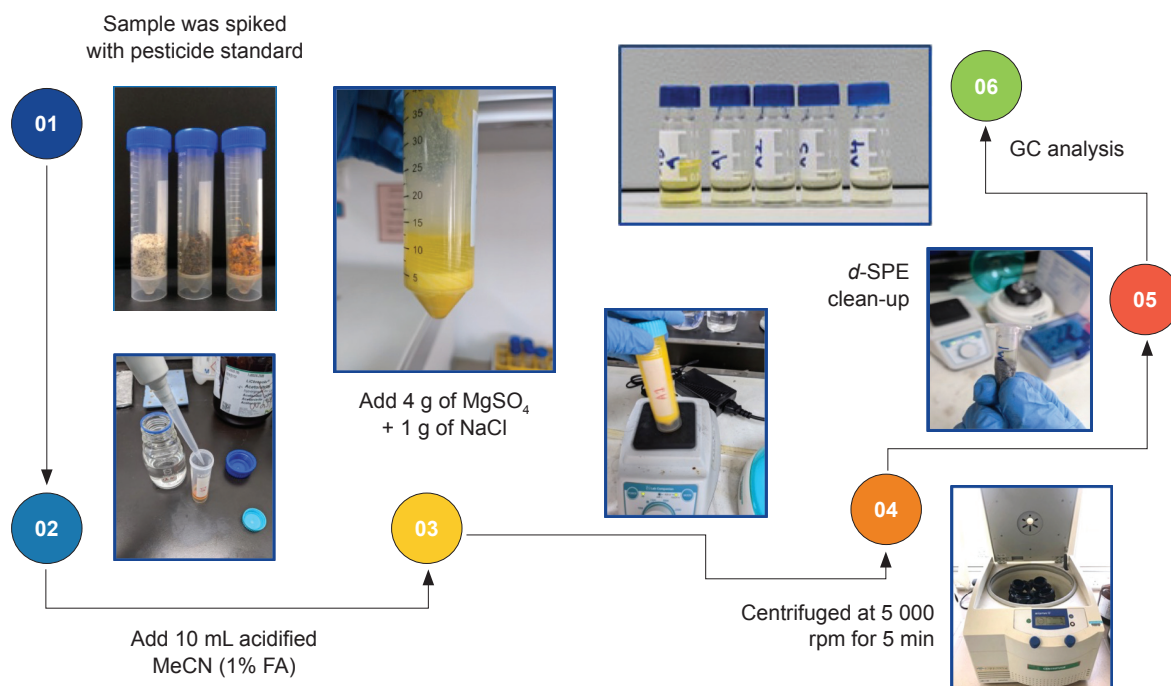


Figure 2. Flow chart of modified QuEChERS procedure.

Finally, 1 mL aliquot was filtered through a 0.22 μm nylon filter into an autosampler vial prior to GC- μECD analysis.

Analytical Method Validation for Pesticide Residual Analysis

In-house validation study was conducted in order to evaluate accuracy, precision, linearity, LOD, LOQ, matrix effects and repeatability as described in Document No. SANTE/2017/11813 (SANTE, 2020).

Statistical Analysis

All analyses were performed in six replications and the results were expressed as mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey's test ($\alpha=0.05$) were used for comparison of the treatments using Minitab 21 Statistical Software (USA).

RESULTS AND DISCUSSION

Linearity, Matrix-Matched Calibration Curve and Matrix Effect

Calibration curves for triazole fungicides were constructed by plotting the individual peak area against the concentration of corresponding calibration standards at 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mg L^{-1} in acetonitrile. A good linearity with the regression coefficients (R^2) of > 0.99 was obtained for all triazole residual. Figure 3 shows chromatogram of the triazole mixture standard analysed using GC- μECD . Matrix effect was calculated by comparing the slope of calibration curves of acetonitrile and matrix. The matrix effects for triazole fungicides in palm kernel,

mesocarp and RAC are listed in Table 1. It can be seen that triazole signal suffered from ion enhancement effect caused by the matrix ($>30\%$ of matrix effect). The highest matrix suppression was observed in RAC when compared to palm kernel and mesocarp. Epoxiconazole showed high matrix suppression in all matrices studied. From this observation, it was decided that further work on method development and validation of triazole fungicides would be based on the matrix-matched calibration curve. Figure 4 shows the calibration curve of tetraconazole in acetonitrile, palm kernel, RAC and mesocarp matrix.

LOD and LOQ

LOD and LOQ of an individual analytical procedure is the lowest amount of analyte presence in a sample which can be detected and quantitatively determined, respectively (European Medicines Agency, 1995). The equations to calculate LOD and LOQ for each triazole analytes are as follows:

$$\text{LOD} = (3.3 \sigma) / S \quad (2)$$

$$\text{LOQ} = (10 \sigma) / S \quad (3)$$

where σ is denoted as the standard deviation of the regression line and S is denoted as the slope of the calibration curve. The LOD and LOQ obtained were in the range between 0.12 and 0.23 mg kg^{-1} and 0.31 and 0.70 mg kg^{-1} , respectively. Chromatogram of triazole analytes in the blank and spiked matrix of palm kernel, mesocarp and RAC are shown in Figure 5. Method validation study was evaluated in terms of apparent recovery, repeatability, linearity, limit of quantification, matrix effects, reproducibility as well as intra-day and inter-day precision as described in Document No. SANTE/11945/2017 (SANTE, 2020).

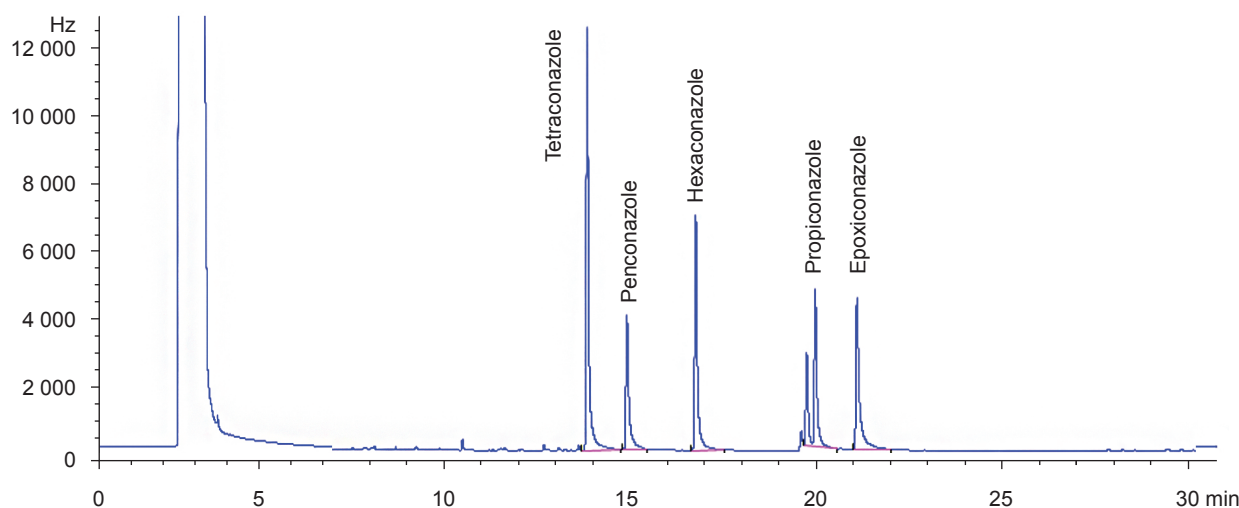


Figure 3. GC- μECD chromatogram of triazole residuals at concentration of 1.0 mg L^{-1} .

TABLE 1. MATRIX EFFECT IN PALM KERNEL, MESOCARP AND RAC

Analytes	Retention time	Palm kernel (%)	Mesocarp (%)	RAC (%)
Tetraconazole	14.14	31.28	26.79	33.52
Penconazole	15.25	39.30	29.63	43.72
Hexaconazole	17.17	54.64	48.69	63.73
Propiconazole	20.08	197.55	197.70	225.04
Epoxiconazole	21.46	288.26	264.71	317.33

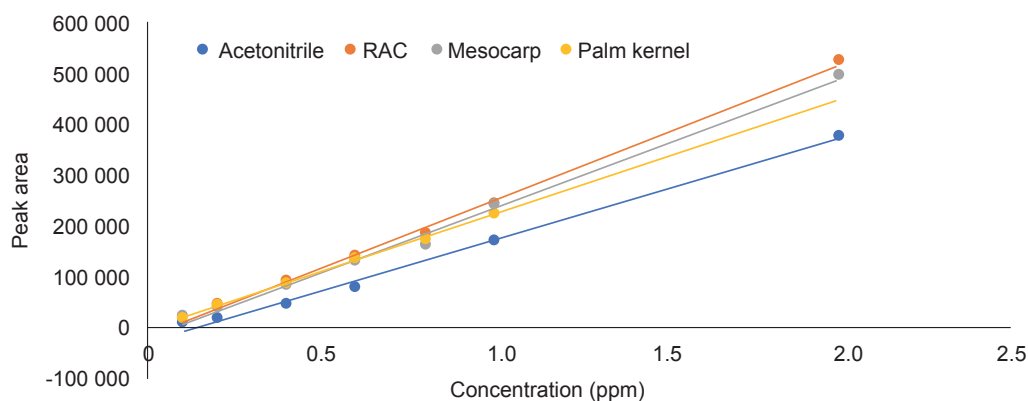


Figure 4. Calibration curve of tetraconazole in acetonitrile, palm kernel, mesocarp and RAC extract.

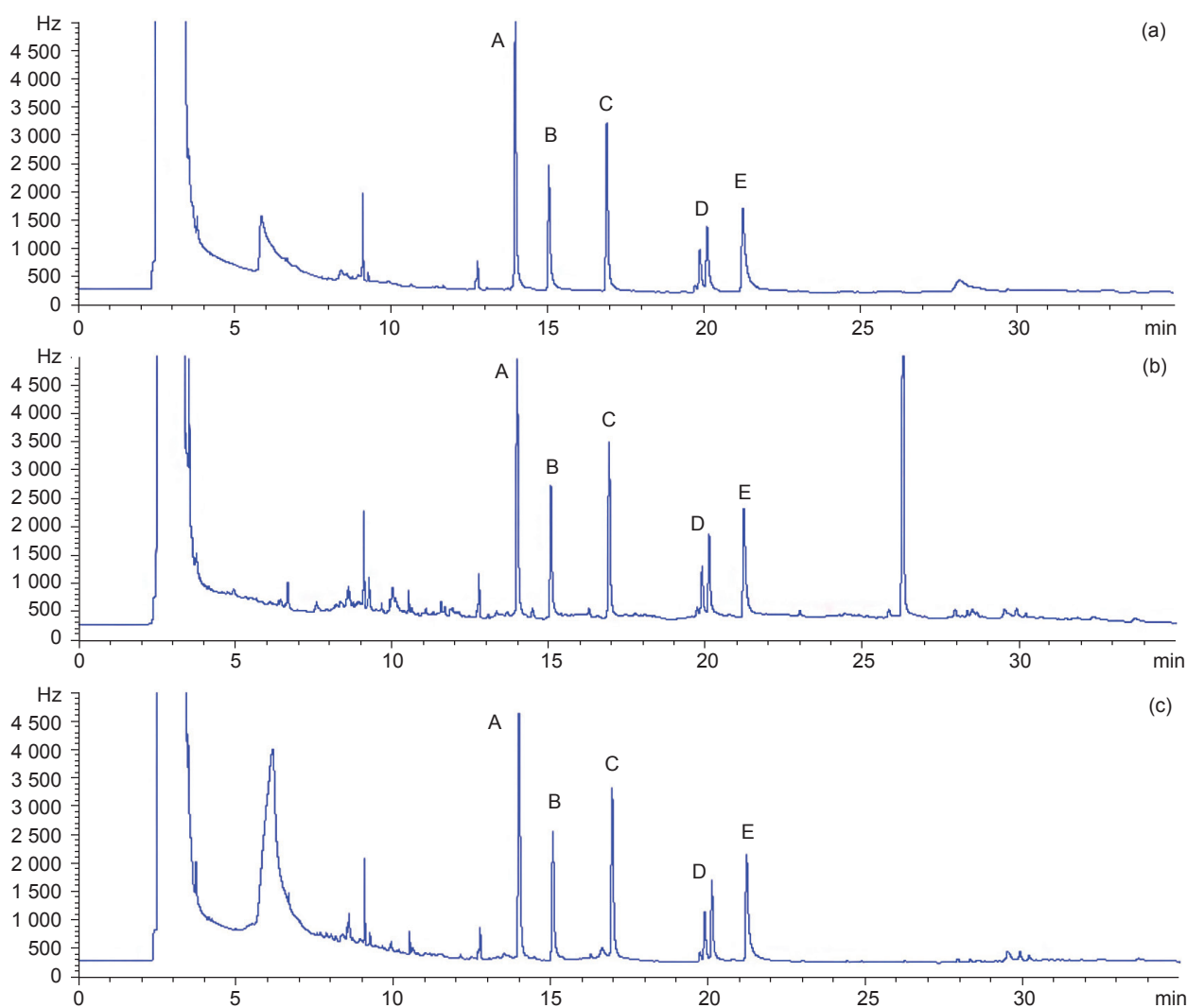


Figure 5. Chromatograms of (A) tetraconazole, (B) penconazole, (C) hexaconazole, (D) propiconazole and (E) epoxiconazole in (a) spiked palm kernel (b) mesocarp and (c) RAC at 0.5 mg kg^{-1} .

Optimisation of Modified QuEChERS

Optimisation of different extraction solvents namely 100.00% acetonitrile, acidified acetonitrile containing 1.00% acetic acid, 3.00% acetic acid, 1.00% formic acid and 3.00% formic acid were investigated to find suitable solvent for pesticide residuals extraction from palm kernel, mesocarp and RAC. For mesocarp matrix, one additional solvent extraction containing 10.00% distilled water was tested. It was indicated from all figures that the acidified acetonitrile with 1.00% acetic acid was the most effective solvent for pesticide residue extraction. Results showed that acidified acetonitrile with 1.00% formic acid gave good recoveries in palm kernel and RAC matrices, with mean recoveries obtained ranged from 81.53% to 96.93%. For mesocarp sample, acidified acetonitrile with 1% acetic acid and 10.00% distilled water gave the highest recoveries in the range of 104.00% to 111.87% with %RSD of less than 6.00%. Addition of water in dry sample is necessary to loosen up the interaction between the analytes and the matrix hence, enhancing separation of the analytes (Santana-Mayor *et al.*, 2019). Numerous studies have combined acetonitrile with other solvents such as formic acid, acetic acid, citric acid, ethyl acetate, methanol, dichloromethane, and hexane to create acidified acetonitrile in order to improve the extraction efficiency (Perestrelo *et al.*, 2019).

Acetonitrile was shown to be the most suitable extraction solvent (Lentza-Rizos *et al.*, 2001). It was reported that acetonitrile is able to extract a wide range of pesticide residues and has higher capacity and selectivity as compared to the other solvents (González-Curbelo *et al.*, 2015; Maštovská and Lehotay, 2004). In addition, acetonitrile offers the possibility of subsequent liquid-liquid clean-up step as it forms distinct partitioning phases with non-polar solvents. Acidified solution is more effective to extract triazole pesticides due to the presence of 1,3-imidazole or 1,2,4-triazole ring which is capable of protonating the molecules of fungicides, and high activity of hydrogen ions in anhydrous solution (Zayats *et al.*, 2015). Wang *et al.* (2017) reported that acidification of acetonitrile with 1% acetic acid showed extraction recovery improvement for all *Alternaria* toxins in Jujube samples. Reichert *et al.* (2018) found that acidified acetonitrile with 1% acetic acid as extraction solvent provides the best extraction efficiency of 117 pesticides and 30 mycotoxins analytes in raw coffee when compared to acidified acetonitrile with 4% of formic acid.

Optimisation of d-SPE

The selection of adsorbents was conducted to assess the effectiveness of the clean-up method. The clean-up step is vital to remove co-extractives

in the samples (Tuzimski and Rejczak, 2016). Prior to injection, aliquot sample was added into d-SPE tubes containing several sorbents and subjected to centrifugation to provide immediate contact between the sample and sorbent and subsequently improving the effectiveness of the clean-up technique (Senes *et al.*, 2020). The final clear aliquot obtained is then injected into GC or LC for quantification.

Table 2 shows the effect of three different ratios of MgSO_4 and PSA on the mean recoveries obtained from spiked PKC. The mean recoveries of d-SPE clean-up in the absence of GCB (45:20:0) was the highest in all analytes when compared to d-SPE clean-up with GCB. For PKC matrix, GCB was not required in d-SPE clean-up due to absence of colouring supernatant. Result showed that increment of PSA ratio had no significant effect on the recovery ($p < 0.05$). Statistical analysis also showed that there was no significant ($p < 0.05$) effect of different ratios of MgSO_4 and PSA on the mean recovery. Thus, it was concluded that the ratio of 45:20 for both MgSO_4 and PSA was found to be the optimum clean-up sorbents ratio proven by the highest recovery for all analytes.

Effect of different ratios of adsorbents; MgSO_4 , PSA and GCB at ratios of 45:20:0, 45:20:20 and 45:25:25 from fortified mesocarp is tabulated in Table 3. After extraction, the supernatant was observed to have high amount of yellowish coloured pigment. The presence of pigment in the aliquot did not contribute to any peaks but it could reduce the half-life of GC column. Hence, GCB was introduced to the treatment to enable removal of colour pigment. Results showed satisfactory mean recovery for all pesticide residual was obtained in the range of 80.52% to 93.15% with %RSD less than 10.00% when the adsorbent ratio was 45:20:20. Similar results were also found for RAC samples where the ratio of 45:20:20 gave acceptable range of recoveries of 81.54% to 93.15% with %RSD less than 5.00% (Table 4). It was also observed that the increment of GCB ratio demonstrated a negative effect on recovery. The mean percentage of recovery was slightly reduced when the portion of GCB increased from 20.00% to 25.00%. The use of PSA and GCB could raise some concerns such as loss of analytes and low recovery especially for certain pesticides (Deme *et al.*, 2014; Muhamad *et al.*, 2012b). Guan *et al.* (2013) agreed that extensive use of adsorbent might affect the recovery of target analytes, since the target analytes could be adsorbed by the sorbent.

Method Accuracy

Recovery studies. Three fortification levels of 0.5, 1.0 and 5.0 mg kg⁻¹ were evaluated for recovery determinations. All recovery experiments were performed in six replicates at each level as suggested

in SANTE document. Tables 5, 6 and 7 show the mean recoveries and %RSD at different concentration levels spiked in palm kernel, mesocarp and RAC, respectively. The mean recoveries ranged from 85.29% to 104.91% with RSD less than 6.00% were observed in palm kernel samples. For mesocarp samples, most of analytes were recovered in the range of 89.95% and 106.28% with RSD of less than 5.00%. For RAC samples, the mean of recoveries was recorded within 80.58% and 108.47% with %RSD of less than 8.00%. Relatively low %RSD obtained from the recovery assessment indicates that the developed method has good repeatability (Khairuddin *et al.*, 2021).

Method Precision

Repeatability. Repeatability is estimated by determining the inter- and intra-day relative standard deviation (RSD, %) by repeated analysis (n=6) of a spiked palm kernel, mesocarp and RAC samples. Two fortification levels of 1.0 mg kg⁻¹ and 5.0 mg kg⁻¹ were tested from run-to-run over 1 and 5 days. The

mean recovery for palm kernel replicates on day 1 and 5 were within 80.32% and 103.99% with %RSD below 6.00%. For mesocarp and RAC matrices, the recoveries for repeatability study performed at day 1 and 5 were ranged within 86.05% and 111.80%, and 97.89% and 107.99%, respectively. The %RSD calculated from recovery assessment are less than 10.00%.

Reproducibility. Reproducibility is assessed by comparing the relative standard deviation of the recovery of spiked samples within the same day (intra-day) by two analysts. Method reproducibility is expressed as mean recovery (%), and relative standard deviation (%RSD) spiked at 1.0 mg kg⁻¹ in palm kernel, mesocarp and RAC. Analyses performed by analyst-2 was found comparable with that of analyst-1. Mean recoveries observed for analyst-1 were within the range of 78.26% and 101.06%. For analyst-2, mean recoveries were found between 80.37% and 92.89%. The %RSD found were less than 10.00% for both analysts.

TABLE 2. EFFECT OF MgSO₄: PSA: GCB RATIO ON MEAN RECOVERY OF PESTICIDES RESIDUAL FROM FORTIFIED PKC AT 1.0 mg kg⁻¹

Pesticide residual	% Mean recovery $\pm$ SD		
	45:20:0	45:25:0	45:30:0
Tetraconazole	93.19 $\pm$ 1.80 ^a	82.30 $\pm$ 1.92 ^a	90.83 $\pm$ 2.50 ^a
Penconazole	92.96 $\pm$ 4.01 ^a	81.48 $\pm$ 1.28 ^a	86.91 $\pm$ 3.02 ^a
Hexaconazole	93.77 $\pm$ 1.27 ^a	78.87 $\pm$ 1.38 ^a	85.98 $\pm$ 3.09 ^a
Propiconazole	93.20 $\pm$ 5.62 ^a	82.70 $\pm$ 0.37 ^a	88.92 $\pm$ 2.35 ^a
Epoxiconazole	89.85 $\pm$ 6.38 ^a	88.20 $\pm$ 3.94 ^a	88.16 $\pm$ 2.96 ^a

Note: Mean values with different letter superscripts indicate significant differences between ratio ($p < 0.05$).

TABLE 3. EFFECT OF MgSO₄: PSA: GCB RATIO ON MEAN RECOVERY OF PESTICIDES RESIDUAL FROM FORTIFIED MESOCARP AT 1.0 mg kg⁻¹

Pesticide residual	% Mean recovery $\pm$ SD		
	45:20:0	45:20:20	45:25:25
Tetraconazole	68.81 $\pm$ 5.32 ^a	80.52 $\pm$ 1.85 ^a	69.01 $\pm$ 4.06 ^a
Penconazole	75.81 $\pm$ 5.70 ^b	93.15 $\pm$ 2.01 ^a	70.22 $\pm$ 4.94 ^b
Hexaconazole	78.83 $\pm$ 6.05 ^b	85.87 $\pm$ 1.84 ^a	67.70 $\pm$ 2.19 ^c
Propiconazole	70.50 $\pm$ 4.51 ^a	81.54 $\pm$ 5.06 ^a	72.48 $\pm$ 5.22 ^a
Epoxiconazole	75.58 $\pm$ 5.77 ^{a,b}	81.25 $\pm$ 4.22 ^a	70.56 $\pm$ 3.68 ^b

Note: Mean values with different letter superscripts indicate significant differences between ratio ($p < 0.05$).

TABLE 4. EFFECT OF MgSO₄: PSA: GCB RATIOS ON MEAN RECOVERY OF PESTICIDES RESIDUAL FROM FORTIFIED RAC AT 1.0 mg kg⁻¹

Pesticide residual	% Mean recovery $\pm$ SD		
	45:20:0	45:20:20	45:25:25
Tetraconazole	69.20 $\pm$ 1.75 ^b	81.94 $\pm$ 2.27 ^a	68.71 $\pm$ 4.06 ^b
Penconazole	73.16 $\pm$ 2.23 ^b	78.77 $\pm$ 2.48 ^a	71.22 $\pm$ 1.69 ^b
Hexaconazole	80.19 $\pm$ 2.80 ^a	78.14 $\pm$ 3.01 ^a	77.27 $\pm$ 1.97 ^a
Propiconazole	71.61 $\pm$ 1.88 ^b	81.04 $\pm$ 3.69 ^a	70.71 $\pm$ 1.45 ^b
Epoxiconazole	68.08 $\pm$ 3.72 ^b	89.58 $\pm$ 3.77 ^a	68.09 $\pm$ 1.97 ^b

Note: Mean values with different letter superscripts indicate significant differences between ratio ($p < 0.05$).

TABLE 5. MEAN RECOVERY (%) AND RSD (%) FOR TRIAZOLE RESIDUES FROM SPIKED PALM KERNEL AT THREE LEVELS OF CONCENTRATIONS

Pesticide residual	Palm kernel					
	0.5 mg kg ⁻¹		1.0 mg kg ⁻¹		5.0 mg kg ⁻¹	
	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD
Tetraconazole	87.78	4.22	94.01	4.52	81.10	2.50
Penconazole	85.29	3.03	94.64	4.44	80.82	3.02
Hexaconazole	87.33	1.36	86.82	4.78	76.31	3.09
Propiconazole	89.22	5.32	88.98	4.63	82.87	2.35
Epoxiconazole	94.32	4.87	94.68	3.10	90.95	2.96

TABLE 6. MEAN RECOVERY (%) AND RSD (%) FOR TRIAZOLE RESIDUES FROM SPIKED MESOCARP AT THREE LEVELS OF CONCENTRATIONS

Pesticide residual	Mesocarp					
	0.5 mg kg ⁻¹		1.0 mg kg ⁻¹		5.0 mg kg ⁻¹	
	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD
Tetraconazole	106.28	0.98	100.55	1.54	86.04	4.36
Penconazole	106.11	0.53	101.48	2.70	83.46	3.11
Hexaconazole	105.49	0.25	101.27	3.24	82.48	4.13
Propiconazole	106.13	0.70	101.70	1.89	84.39	3.21
Epoxiconazole	100.85	4.48	99.06	4.48	87.57	2.87

TABLE 7. MEAN RECOVERY (%) AND RSD (%) FOR TRIAZOLE RESIDUES FROM SPIKED RAC AT THREE LEVELS OF CONCENTRATIONS

Pesticide residual	RAC					
	0.5 mg kg ⁻¹		1.0 mg kg ⁻¹		5.0 mg kg ⁻¹	
	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD
Tetraconazole	101.20	1.20	86.30	2.58	95.21	3.92
Penconazole	88.28	4.11	81.53	2.70	80.93	4.27
Hexaconazole	108.47	1.71	82.54	3.17	101.41	2.04
Propiconazole	80.58	1.72	90.13	4.73	89.61	4.72
Epoxiconazole	81.47	4.01	96.93	3.42	92.99	4.84

CONCLUSION

A simple, fast and reliable method from modified QuEChERS approach was successfully developed and optimised for the determination of triazole residues in palm kernel, mesocarp and RAC matrices. The method is successfully validated in-house, and fulfil the requirements of important validation parameters such as linearity, recovery study and method precision (repeatability and reproducibility). The LOD and LOQ fell between 0.12-0.23 mg kg⁻¹ and 0.31-0.70 mg kg⁻¹, respectively. From this work, the developed method can be used to determine the level of triazole fungicides residues in palm kernel, mesocarp and RAC matrices in meeting the standard legislative limits of importing countries.

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MOLECULAR APPROACHES IN ASSESSING THE SOIL MICROBIAL DIVERSITY OF MALAYSIAN OIL PALM PLANTATIONS: A REVIEW

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ABSTRACT

Soil microorganisms are vital to the overall function, stability and sustainability of the ecosystem. Microbial communities are key bio-indicators for sustainable agro-ecosystem management and for evaluating oil palm soil health in Malaysia. The development of effective methods for studying the diversity, distribution and behaviour of soil microorganisms is essential for a broader understanding of soil quality and fertility. In the past, culture-dependent, Denaturing Gradient Gel Electrophoresis (DGGE) and Terminal-Restriction Fragment Length Polymorphism (T-RFLP) were used to assess soil microbial community, abundance and diversity in the oil palm soils. However, these methods have significant shortcomings and limitations in microbial ecology monitoring. In recent years, high-throughput next-generation sequencing has dramatically increased the resolution and detectable spectrum of microbial from environmental samples, and has played a significant role in microbial ecology studies. Next-generation sequencing using 454-pyrosequencing, Ion Torrent, Illumina and PacBio platforms are rapid and flexible. These sequencing platforms are powerful metagenomic tools for analysing microbial communities, including their structure, metabolic potential, diversity and their impact on ecosystem functions. In this review, the major development of molecular approaches used in the characterisation of soil microbial community dynamics and their functions in Malaysian oil palm plantations are also discussed.

Keywords: culture-dependent, culture-independent, microbial diversity, oil palm plantation.

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INTRODUCTION

Soil is one of the main reservoirs of biodiversity which consists of complex and diverse species of living organisms (Bardgett and Van Der Putten, 2014). Soil supports a large fraction of below-ground organisms ranging from macrofauna (earthworms, woodlice, ants, termites, beetles, arachnids), mesofauna (mites, springtails, enchytraeids) to microscopic organisms such as protozoa, bacteria, fungi and archaea (Food and Agriculture Organization, 2020). Among these organisms, soil microorganisms play a key role in regulating ecosystem functions and services such as biogeochemical cycles, organic

matter decomposition, plant productivity and greenhouse gas emissions (GHG) (Bardgett and Van Der Putten, 2014; Delgado-Baquerizo *et al.*, 2016). These organisms regulate climate change through the emission of atmospheric GHGs, such as carbon dioxide, methane and nitrous oxide. Their roles are particularly crucial in agricultural ecosystems to enable sustainable farming, food security and climatic impacts (Singh *et al.*, 2010).

The year 2017 was a historical milestone for the Malaysian oil palm industry as it marks 100th year celebration of commercial oil palm planting in this country. Oil palm (*Elaeis guineensis* Jacq.) cultivation occupies over 5.67 million hectares of land by 2022 and impacted the country's economy in the last 100 years (Parveez *et al.*, 2023). In 2022, Malaysia became the major exporter of palm oil, as reflected by the 24.77 million tonnes of the global oils and fats exported, while contributing to 57.9% of the total

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palm oil trade (Parveez *et al.*, 2023). Maintaining oil palm plantations through regular harvesting, pruning, fertilisation and pest management have resulted in significant alterations to the environment and microclimate (Dhandapani *et al.*, 2020). Various studies have suggested that plantation agriculture would affect the microbial composition in soils of tropical regions (Lee-Cruz *et al.*, 2013; McGuire *et al.*, 2015).

According to Food and Agriculture Organization (2020), a gram of soil contains up to one billion bacterial cells, and 200 m of fungal hyphae belonging to thousands of taxa. Therefore, it is crucial to identify and characterise these diverse microorganisms with appropriate methods and tools. Traditionally, methods such as isolation, inoculation, and cultivation of pure cultures from soils were used many years ago (Dobrovolskaya *et al.*, 2009). Through these methods, soil microbes can be distinguished based on colony morphology characterisation, which would provide a solution for microbial identification. However, there are microbes' group or taxa that are unable to grow under laboratory conditions and selective media, which would not have been accounted for in the diversity (Stewart, 2012). Thus, these methods will only be able to identify a minor proportion of the total microbial community and do not reflect the whole microbial diversity in soil (Dobrovolskaya *et al.*, 2009).

The use of molecular approaches has been applied to quantify the diversity and identity of soil microbial communities. Application of genes such as 16S rDNA (bacteria, archaea), 18S rDNA (eukaryotes) and ITS (fungi) were employed in Terminal-Restriction Fragment Length Polymorphisms (T-RFLP), Temperature Gradient Gel Electrophoresis (TGGE) and Denaturing Gradient Gel Electrophoresis (DGGE) (Prosser, 2002). These PCR-based genetic fingerprinting techniques provide microbial profiling based on the variation of targeted genes amplified from the soil (Anderson and Cairney, 2004). These techniques are reproducible and effective at characterising the dynamics of microbial diversity, including uncultivated microbes (Ayob and Kusai, 2021; Gryta and Frac, 2020). Nevertheless, these methods

have limitations as they are very laborious, expensive with low throughput (Nkongolo and Narendrula-Kotha, 2020).

The recent development of molecular tool has facilitated in measuring of soil microbial community and diversity. The most useful approach involves sequencing ribosomal genes which are highly conserved and can reveal community structure across the taxa such as prokaryotes (bacteria, archaea) and eukaryote (fungi) (Nkongolo and Narendrula-Kotha, 2020). One of the promising approaches to studying complex soil microbial communities is using next-generation sequencing (NGS). The revolution of NGS technologies is demonstrated through a variety of platforms such as 454 pyrosequencing, Ion Torrent, Illumina MiSeq, HiSeq and NovaSeq, PacBio and Oxford Nanopore MinION (Nilsson *et al.*, 2019). These platforms provide comprehensive information in detecting microbial communities and their functional ecology over large spatial and temporal scales. Advancements in molecular methods has allowed for a higher resolution assessment of microbial compositions (Dom *et al.*, 2021).

Furthermore, the analysis of diversity can be conducted by considering factors that influence the biodiversity of soil microbial communities and its impact on oil palm management practices, and predicting ecosystem responses to a changing environment (Ayob and Kusai, 2021; Tripathi *et al.*, 2012). There have been several studies that investigated the effects of land use change on the microbial community in the Malaysian oil palm plantation ecosystem using traditional and advance molecular methods, as listed in Table 1. In this article, we briefly describe the major development in molecular methodological strategies to better assess and understand the distribution and characterisation of soil microbial diversity in Malaysian oil palm plantation ecosystems. Figure 1 briefly demonstrates both culture-dependent and -independent approaches for identifying soil microbial communities. In particular, we also identify the potential function of each microbial species in oil palm plantations using molecular approaches, as summarised in Table 2.

TABLE 1. MOLECULAR APPROACHES USED TO IDENTIFY AND CHARACTERISE SOIL MICROBIAL COMMUNITIES IN MALAYSIAN OIL PALM PLANTATIONS

Method	Microorganism	Locality	Age of oil palm	References
454-pyrosequencing (Roche)	Bacteria	Peninsular Malaysia	ND	Tripathi <i>et al.</i> (2012)
Illumina HiSeq	Bacteria	Sabah	20 to 30-yr-old	Lee-Cruz <i>et al.</i> (2013)
454-pyrosequencing (Roche)	Fungi	Sabah	20 to 30-yr-old	Kerfahi <i>et al.</i> (2014)
Illumina MiSeq	Fungi	Negeri Sembilan	25-yr-old	McGuire <i>et al.</i> (2015)
Culture-dependent	Fungi	Sarawak	7 and 11-yr-old	Ahmad Ali <i>et al.</i> (2016)

TABLE 1. MOLECULAR APPROACHES USED TO IDENTIFY AND CHARACTERISE THE SOIL MICROBIAL COMMUNITIES IN MALAYSIAN OIL PALM PLANTATIONS (continued)

Method	Microorganism	Locality	Age of oil palm	References
Illumina HiSeq	Bacteria, eukaryotes, archaea, and functional gene	Sabah	20 to 30-yr-old	Tripathi <i>et al.</i> (2016)
Illumina MiSeq	Bacteria	Negeri Sembilan	25-yr-old	Wood <i>et al.</i> (2017)
Culture-dependent	Fungi	Sarawak	3.5-yr-old	Ayob <i>et al.</i> (2018)
Culture-dependent	Fungi	Sarawak	7.5 and 11.5-yr-old	Kusai <i>et al.</i> (2018)
Denaturing Gradient Gel Electrophoresis (DGGE)	Bacteria	Sarawak	7 -yr-old	Maidin <i>et al.</i> (2018)
454-pyrosequencing (Roche)	Eukaryotes	Sarawak	7 and 11-yr-old	Ahmad Ali <i>et al.</i> (2018)
Illumina MiSeq	Bacteria	Sabah	10-yr-old	Tin <i>et al.</i> (2018)
Denaturing Gradient Gel Electrophoresis (DGGE)	Bacteria	Sarawak	1.5 – 5.5-yr-old	Ahamed Bakeri <i>et al.</i> (2019)
Culture-dependent	Bacteria	Sarawak	7.5-yr-old	Kusai and Ayob, 2020
Illumina MiSeq	Fungi	Sabah	7-yr-old	Robinson <i>et al.</i> (2020)
Illumina HiSeq	Bacteria	Kelantan	ND	Rupert <i>et al.</i> (2020)
Culture-dependent; Denaturing Gradient Gel Electrophoresis (DGGE)	Bacteria	Sarawak	12-yr-old	Ayob and Kusai, 2021
Illumina MiSeq	Bacteria, eukaryotes, fungi	Sabah	2, 7 and 12-yr-old	Drewer <i>et al.</i> (2021)
Culture-dependent; Denaturing Gradient Gel Electrophoresis (DGGE); Illumina MiSeq	Bacteria	Negeri Sembilan	25-yr-old	Salamat <i>et al.</i> (2021)
Culture-dependent; Denaturing Gradient Gel Electrophoresis (DGGE)	Fungi	Sarawak	3 to 4-yr-old	Wong <i>et al.</i> (2021)

Note: ND - Not determined; yr - year.

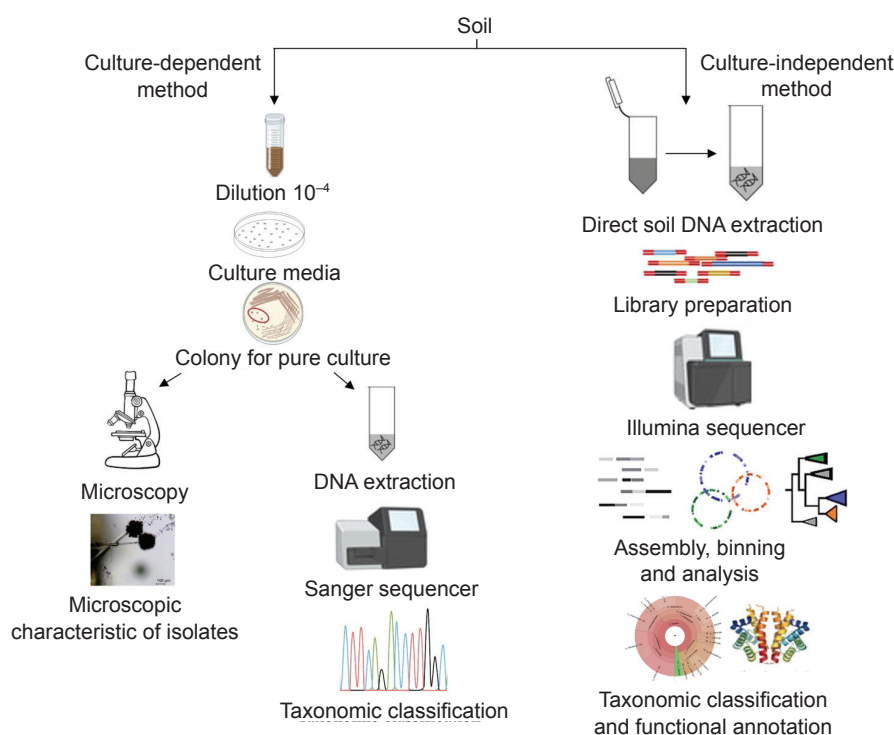


Figure 1. Schematic diagram of culture-dependent and culture-independent methods for identifying soil microbial communities.

TABLE 2. SUMMARY OF POTENTIAL FUNCTIONS OF EACH SPECIES IDENTIFIED IN OIL PALM PLANTATIONS USING MOLECULAR APPROACHES

Method	Phylum	Order/ Family/ Species	Function/ role	References
Culture-dependent	Actinobacteria	<i>Micrococcus</i> sp.	i. Degrades lignocellulose ii. Plant-growth promoting bacteria	Ayob and Kusai, 2021
		<i>Arthrobacter</i> sp.	i. Ability to remove atrazine herbicide in soil ii. Remediation of atrazine contaminated soil	Kusai and Ayob, 2020
	Firmicutes	<i>Bacillus cereus</i>	i. Nitrogen-fixing bacteria	Kusai and Ayob, 2020
		<i>Bacillus subtilis</i>	i. Produce cellulose-degrading enzymes to hydrolyse cellulose of plant litter	Kusai and Ayob, 2020
	Proteobacteria	<i>Burkholderia</i> sp.	i. Involve in the decaying process of the plant litter ii. Ability to breakdown lignin	Ayob and Kusai, 2021; Kusai and Ayob, 2020
		<i>Burkholderia cepacia</i>	i. Biocontrol agent to inhibit <i>Ganoderma boninense</i> , the causal agent of basal stem rot of oil palm ii. Improve plant growth	Kusai and Ayob, 2020
	Ascomycota	<i>Aspergillus niger</i>	i. Enhance phosphate solubilisation process in agricultural soils ii. Increase fertility of soil and oil palm health	Kusai <i>et al.</i> (2018)
		<i>Penicillium chrysogenum</i>	i. Ability to degrade wood in soils	Kusai <i>et al.</i> (2018)
		<i>Penicillium</i> sp.	i. Produce cellulases for degradation of plant biomass	Kusai <i>et al.</i> (2018)
		<i>Trichoderma atroviride</i>	i. Produce laccases for degradation of lignocellulosic materials	Kusai <i>et al.</i> (2018)
		<i>Trichoderma harzianum</i>	i. Possess biocontrol properties against basal stem rot disease of oil palm ii. Incorporate in biofertiliser to reduce disease incidence in oil palm plantation	Kusai <i>et al.</i> (2018)
		<i>Talaromyces purpurogenus</i>	i. Involve in phosphate solubilisation in soil	Kusai <i>et al.</i> (2018)
		<i>Trichoderma koningii</i>	i. Involve in nitrous oxide (N ₂ O) production	Kusai <i>et al.</i> (2018)
	Actinobacteria	-	i. Breakdown macromolecules such as cellulose, lignin, chitin and starch by producing extracellular enzyme	Maidin <i>et al.</i> (2018)
		<i>Actinomycetales</i> , <i>Solirubrobacterales</i>	i. Potential biomarker in responses to land disturbance	Ahamed Bakeri <i>et al.</i> , 2019; Lee-Cruz <i>et al.</i> , 2013; Salamat <i>et al.</i> , 2021
		<i>Streptomyces</i> spp.	i. Synthesise antibiotics in the presence of plant pathogens ii. Mediating species interactions in soil	Ahamed Bakeri <i>et al.</i> (2019)
		<i>Sinomonas humi</i>	i. Amylolytic bacteria ii. Utilise carbon sources	Tin <i>et al.</i> (2018)
	Acidobacteria	-	i. Decompose lignocellulosic plant biomass	Ahamed Bakeri <i>et al.</i> (2019)
		Frankiales, Pseudonocardiales	Nitrogen fixing bacteria	Ahamed Bakeri <i>et al.</i> (2019)
	Bacteroidetes	-	i. Utilise available organic matter under agriculture soils ii. Cellulose degradation	Ahamed Bakeri <i>et al.</i> (2019)

TABLE 2. SUMMARY OF POTENTIAL FUNCTIONS OF EACH SPECIES IDENTIFIED IN OIL PALM PLANTATIONS USING MOLECULAR APPROACHES (continued)

Method	Phylum	Order/ Family/ Species	Function/ role	References
	Bacteroidetes	<i>Chryseobacterium</i>	i. Plant-growth promoting bacteria	Ayob and Kusai, 2021
	Chloroflexi	-	i. Involve in nitrogen cycle such as nitrogen fixation and nitrite oxidation	Ahamed Bakeri <i>et al.</i> (2019)
	Cyanobacteria	-	i. Plant-growth promoting bacteria ii. Utilise nitrogen through nitrogen fixation process iii. Increase phosphorus availability iv. Photosynthetic bacteria	Ahamed Bakeri <i>et al.</i> (2019)
	Firmicutes	<i>Paenibacillus polymyxa</i> , <i>Paenibacillus jamilae</i> , <i>Paenibacillus peoriae</i>	i. Involve in nitrogen-fixation process ii. Biocontrol agent	Maidin <i>et al.</i> (2018)
	Nitrospirae	Nitrospirales	i. Involve in nitrification activity in soils	Ahamed Bakeri <i>et al.</i> , 2019; Maidin <i>et al.</i> , 2018; Tripathi <i>et al.</i> , 2016
	Proteobacteria	<i>Rhodoplanes</i>	i. Plant growth promoting bacteria	Maidin <i>et al.</i> (2018)
		<i>Rhizobium</i> sp.	i. Plant growth promoting bacteria ii. Nitrogen-fixing bacteria	Maidin <i>et al.</i> (2018)
		α -Proteobacteria	i. Degrade inorganic compounds ii. Involve in nitrogen-fixation process	Ahamed Bakeri <i>et al.</i> (2019)
		β -Proteobacteria; <i>Burkholderia</i>	i. Involve in nitrogen-fixation process	Ahamed Bakeri <i>et al.</i> (2019)
		β -Proteobacteria <i>Nitrosomonadales</i>	i. Ammonia-oxidising bacteria	Ahamed Bakeri <i>et al.</i> (2019)
		γ -Proteobacteria	i. Decompose soil carbon substrate ii. Increase supply of labile carbon substrates to soil	Ahamed Bakeri <i>et al.</i> (2019)
		δ -Proteobacteria	i. Bacterial indicator of land-use change	Ahamed Bakeri <i>et al.</i> (2019)
	Ascomycota	<i>Polyporales</i>	i. Saprophytic fungi growing on fallen woods and grasses	Wong <i>et al.</i> (2021)
		Euratioles, Hypocreales, Sordariales	i. Involve in denitrification process in soil	Wong <i>et al.</i> (2021)
		Xylariales	i. Wood inhabiting saprotrophs	Wong <i>et al.</i> (2021)
		Sebacinales	i. Endophytic fungi ii. Enhance plant growth and disease resistance	Wong <i>et al.</i> (2021)
	Basidiomycota	<i>Agaricales</i>	i. Degrades plant-derived cellulose ii. Lignicolous fungi	Kerfahi <i>et al.</i> (2014)
		Sporidiobolales	i. Saprophytic and mycorrhizal fungi	Kerfahi <i>et al.</i> (2014)
	Glomeromycota	-	i. Arbuscular mycorrhizal fungi ii. Involve in nutrient cycling iii. Involve in soil aggregation	Ahmad Ali <i>et al.</i> (2018)

CULTURE-DEPENDENT APPROACH

Traditional culture-dependent methods are highly determined by on the medium used for isolation, incubation condition and microscopic colonial morphology to identify the microbes (Jackson *et al.*, 2013). Although these methods are easy, fast and reliable for identifying the most prevalent culturable microbial taxa, they have

a number of drawbacks that impede a proper microbial diversity assessment. Processing cultures can be time-consuming and laborious when dealing with a large number of isolates and during these processes, the risk of culture contamination is always high (Jeewon and Hyde, 2007). Moreover, this method provides limited information on microbial communities because more than 99% soil microorganisms remain

'unculturable' in the laboratory as they require specific growth conditions. As a result, a large proportion of microbial communities remains unexplored, and organisms such as Acidobacteria are underrepresented in isolated cultures (Ayob and Kusai, 2021; Liu *et al.*, 2006) when compared to the molecular methods. The molecular methods that describe communities dominated by predominantly uncultivated microorganisms' groups that are not detected by culture, provide a more rapid result, less time-consuming, and been shown to have great flexibility in terms of gene target and target region (Montalvo *et al.*, 2014; Rudkjøbing *et al.*, 2016). A further drawback to the culture-dependent method when predicting fungal community is that some slow-growing species may be overlooked, while the abundance of species with high sporulating rates may be over-estimated (Table 3) (Ayob *et al.*, 2018; Jeewon and Hyde, 2007). Combining culture-dependent and molecular methods, such as PCR-based amplification of rDNA genes and Sanger sequencing, would provide reliable results of identification and would resolve the taxonomic relationship among taxa compared to morphological characterisation alone (Ayob *et al.*, 2018; Kusai *et al.*, 2018). These techniques have resulted in isolation of only the most common and abundant fungi such as asexual ascomycetes *Aspergillus* (Figures 2a-c), *Penicillium* (Figures 2d-2f), and *Trichoderma* (Figures 2g-2i) (Ayob *et al.*, 2018; Kusai *et al.*, 2018) as compared to using the culture-independent technique. For microscopic characterisation, *Aspergillus* sp. had pale brown radiate conidial heads (Figure 3a) while the conidia were globose and found in clusters (Figures 3b-3c). *Penicillium* sp. produced branch conidiospores (Figure 3d) and conidia formed in globose shaped clusters (Figures 3e-3f). Meanwhile, *Trichoderma* sp. produced branched conidiophores that grew from the main hyphae while stout phialides were formed from the branch (Figure 3g) with alobose shaped phialospores (Figures 3h-3i). Most of the isolated fungi phyla are regarded as cellulolytic fungi that are able to produce cellulases for degradation of biomass (Vaishnav *et al.*, 2018). Filamentous fungi such as *Aspergillus niger* (Figure 2a) can enhance phosphate solubilisation in agricultural soils (Li *et al.*, 2016) and are promising microorganisms to be used in oil palm plantations to increase fertility of the soil and oil palm health (Acevedo *et al.*, 2014). On the other hand, *Trichoderma harzianum* (Figure 2h) has biocontrol properties against devastating oil palm disease; basal stem rot caused by *Ganoderma boninense*, and has widely been used as a biofertiliser to reduce disease incidence in oil palm plantations (Naher *et al.*, 2012; Sundram *et al.*, 2008). Meanwhile, the most isolated bacteria in the oil palm plantations of Sarawak are Proteobacteria, Firmicutes, Actinobacteria,

and Bacteroidetes (Ayob and Kusai, 2021; Kusai and Ayob, 2020). Isolated bacteria *Burkholderia cepacia* (class Betaproteobacteria) has been used as a biocontrol agent to reduce incidence of basal stem rot in oil palm by 42%. Members belonging to Firmicutes (class Bacilli), such as *Bacillus cereus* and *B. subtilis*, were commonly isolated from oil palm planted in peat soils (Kusai and Ayob, 2020). *Bacillus cereus* plays an important role as a nitrogen-fixing bacteria (Karagöz *et al.*, 2012), while *B. subtilis* is a cellulolytic bacteria which helps in the hydrolysis process of cellulosic material of plant litter (Kim *et al.*, 2012). Direct microscopic counts of microbes in soil samples, recoverable colony forming units, and sequencing of 16S rDNA, 18S rDNA and ITS genes are used to characterise and better understand the nature of the microbial community composition from oil palm plantation ecosystems associated with agricultural practices and environmental conditions.

CULTURE-INDEPENDENT APPROACHES

Denaturing Gradient Gel Electrophoresis (DGGE)

Recent studies involving identification and characterisation of microbial communities in environmental samples have focused on the use of methods based on genetic diversity that do not require cultivation. This method is known as culture-independent. Theoretically, DNA sequences which differ by even one base-pair can be separated across temperature gradients by Denaturing Gradient Gel Electrophoresis (DGGE). The advantages of DGGE include reliability, reproducibility and the capacity to process multiple samples rapidly by analysing bands that migrate separately on DGGE gels (Ayob and Kusai, 2021; Fakruddin and Mannan, 2013). However, this method is often limited to the low-throughputs from Sanger sequencing, PCR biases, laborious sample handling, and only detects dominant species (Table 3) (Muyzer, 1999; Nkongolo and Narendrula-Kotha, 2020). A study by Miguel *et al.* (2010) suggested that this method possibly detected multiple banding patterns attributed to sequence heterogeneity between copies of 16S rDNA strains, and one band may not necessarily represent one species. Recent studies have reported that unculturable bacteria were the dominant group in the study sites, followed by Proteobacteria, Acidobacteria, Firmicutes and Actinobacteria (Ahamed Bakeri *et al.*, 2019; Ayob and Kusai, 2021; Maidin *et al.*, 2016). The abundance of Acidobacteria in oil palm planted on peat soil suggested that due to the acidic environments, these bacteria grow under oligotrophic conditions. This is consistent with the results obtained from the boreal forest soils (Chapman *et al.*, 2017; Sun *et al.*,

2014; Tsitko *et al.*, 2014). In addition to that, phylum Acidobacteria was only detected using culture-independent methods (Ayob and Kusai, 2021). The fertiliser application increased in certain bacterial communities such as Firmicutes, in a 1.5-year-old oil palm plantation and this application also increased soil pH and phosphate content (Ahamed Bakeri *et al.*, 2019). Firmicutes that thrive in oil palm soils, such as *Bacillus* and *Paenibacillus*, have been reported to be involved in the denitrification of nitrate to nitrogen in soil (Kusai and Ayob, 2020), and have antagonistic properties towards fungal plant pathogens (Maidin *et al.*, 2018). At the genus level, *Nitrospirales* (phylum Nitrospirae) was found in a 3.5-year-old oil palm plantation as nitrifiers correlated with nitrogen input compared to rainforest soil (Ahamed Bakeri *et al.*, 2019). Similarly, the PCR-DGGE profile in the study conducted by Salamat *et al.* (2021) indicated that the oil palm soil with 25 years of fertiliser application was dominated by organisms belonging to the phyla Actinobacteria, Firmicutes and Proteobacteria. The differential responses of bacterial groups may indicate changing environmental variables and reflect the shifting of ecological conditions, such as nitrogen availability and organic carbon, due to long-term fertilisation in mineral soils (Salamat *et al.*, 2021; Sun *et al.*, 2014).

Next-Generation Sequencing (NGS)

Deep sequencing and its capacity to sequence multiple samples simultaneously make metagenomic sequencing very attractive for exploring microbial species in agriculture, ecology and human health. NGS is a high-throughputs

sequencing technology and can be conducted at low analytical cost to assess microbial communities at high resolution (Fierer, 2017). The revolution of NGS technologies from 'second' to 'third' generation sequencing can be seen in a variety of platforms such as 454-pyrosequencing, Ion Torrent, Illumina MiSeq, HiSeq and NovaSeq, PacBio and Oxford Nanopore MinION. These technologies allow data processing and analysing through bioinformatics pipelines more user-friendly and accessible, but still require high expertise and proficiency (Fierer *et al.*, 2021; Nilsson *et al.*, 2019). Besides that, these platforms have provided comprehensive information in detecting microbial communities and their functional ecology over large spatial and temporal scales (Nilsson *et al.*, 2019).

454-Pyrosequencing

Pyrosequencing is a sequencing method that utilises a four-enzyme technology to monitor DNA synthesis via bioluminescence detection (Ahmadian *et al.*, 2006). Although discontinued in 2016, 454-pyrosequencing was the first 'next' generation sequencing system and utilised a novel approach, namely, the detection of pyrophosphate, a by-product of nucleotide incorporation (Saleem, 2020; Slatko *et al.*, 2018). 454-pyrosequencing (Roche) was superior as it was high-throughput, quick, takes only 10 hr from start to finish, and generated long read lengths (up to 700 bp) with 99% accuracy after only 4 hr of run. However, the major drawbacks included high cost of reagents and consumables, and high error rates in homopolymer repeats (Table 3) (Liu *et al.*, 2012; Slatko *et al.*, 2018). Based on previous studies by Kerfahi *et al.* (2014) and Ahmad Ali *et al.* (2018),

TABLE 3. ADVANTAGES AND DISADVANTAGES OF MOLECULAR-BASED METHODS FOR IDENTIFYING SOIL MICROBIAL COMMUNITIES

Method	Advantages	Disadvantages
Culture-dependent	- Fast - Inexpensive - Ease in handling - Ability to further analyse colonies	- Low resolution - Unculturable microbes not detected - Unable to grow under laboratory condition - Lack of expertise and experience on taxonomy and morphological characteristics - Bias towards fast-growing species - Bias towards high sporulating rate species
Denaturing Gradient Gel Electrophoresis (DGGE)	- Direct extraction from soil - No culturing of microorganisms required - Rapid, reliable and reproducible - Large number of samples can be analysed simultaneously	- Laborious - Low throughput - Dependent on DNA extraction efficiency - PCR biases present - One band represent more than one species - Only detects dominant species
454-pyrosequencing	- Longer reads - Short run time - Does not require cloning step	- Expensive reagents and consumables - Error rate with polybase more than six
Illumina sequencing	- High yield - Short run time and scalable - All-in-once analysis in high-throughput - High potential for comparative studies	- Low number of total reads - High instrument cost - Require expertise, high proficiency and training

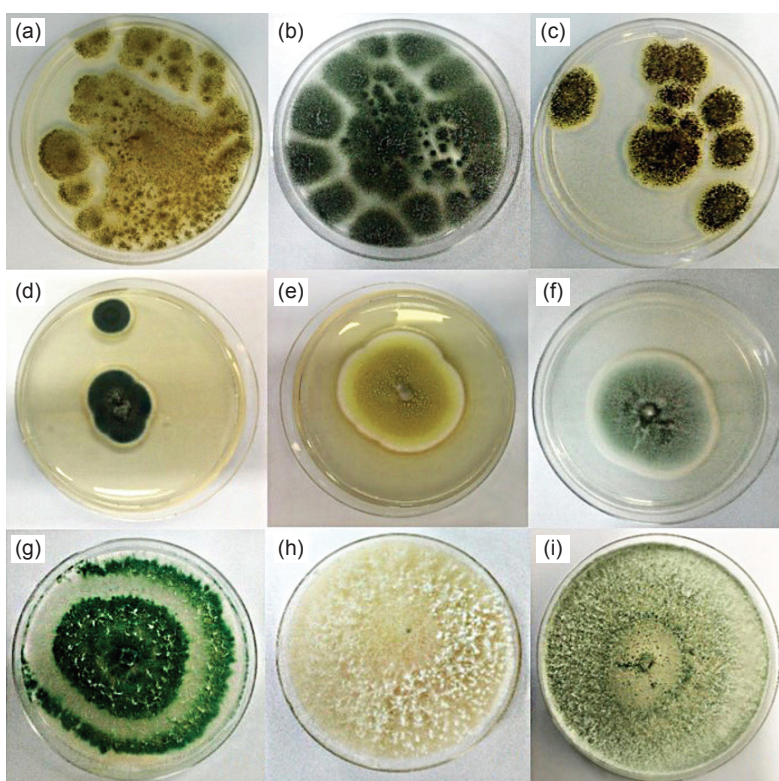


Figure 2. Colony characteristics of fungal cultures isolated from oil palm soil on plate. (a) *Aspergillus niger*, (b) *Aspergillus fumigatus*, (c) *Aspergillus sp.*, (d) *Penicillium freii*, (e) *Penicillium commune*, (f) *Penicillium sp.*, (g) *Trichoderma koningii*, (h) *Trichoderma harzianum* and (i) *Trichoderma sp.*

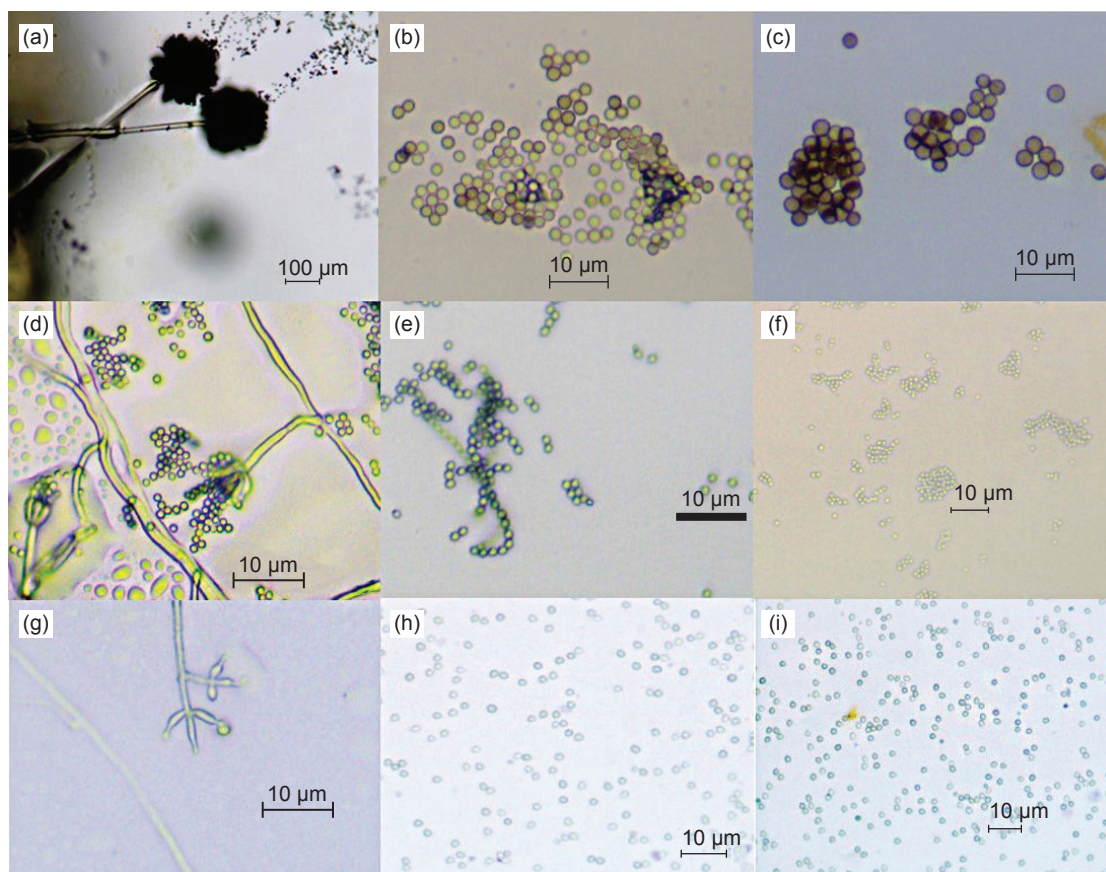


Figure 3. Microscopic characteristics of fungal isolated from oil palm soil on plate. *Aspergillus sp.*: (a) Conidial head, (b, c) Conidia. *Penicillium sp.*, (d) Conidiophore bearing conidiospores, (e, f) Conidia. *Trichoderma sp.*, (g) Phialides, (h, i) Phialospores.

Ascomycota and Basidiomycota dominated the oil palm plantations in Sabah and Sarawak, respectively. The impact of land use change of fungal communities (β -diversity) in Borneo mineral soils resulted in spatial homogenisation of fungal communities in oil palm plantations compared to forests (Kerfahi *et al.*, 2014). In contrast, in the same land use change and sites in Borneo, there was an increase in β -diversity of bacteria detected using Illumina HiSeq in logged over and converted to oil palm plantings (Lee-Cruz *et al.*, 2013). However, the impact of agricultural activities such as water management system resulted in physical, chemical, and biological changes in tropical peatland ecosystem. This in turn led to an increase in eukaryotic diversity in operational taxonomic unit (OTU), α -diversity and total number of phylotypes in oil palm plantations and logged forests (Ahmad Ali *et al.*, 2018). A study by Tripathi *et al.* (2012) observed that the dominant taxa in oil palm plantation were Acidobacteria, Proteobacteria, Chloroflexi and Actinobacteria. The bacterial community composition and diversity was strongly correlated with soil properties, especially soil pH, total carbon, and C/N ratio across land use types such as primary forest, logged forest, and agriculture and pasture lands (Tripathi *et al.*, 2012).

Illumina Sequencing

Illumina sequencing technology was introduced in 2006, and it generates a larger amount of data per run in a more cost-effective manner. The Illumina utilises a sequencing-by-synthesis (SBS) approach, and it differs from 454-pyrosequencing (Nkongolo and Narendrula-Kotha, 2020). However, there are still some limitations in using this technology, as described in Table 3. Illumina amplicon sequencing (HiSeq and MiSeq) has become recently the most popular NGS method used for analysis of microbial diversity and functions in Malaysian oil palm plantations as documented in many studies (Drewer *et al.*, 2020; Lee-Cruz *et al.*, 2013; McGuire *et al.*, 2015; Robinson *et al.*, 2020; Rupert *et al.*, 2020; Salamat *et al.*, 2021; Tin *et al.*, 2018; Tripathi *et al.*, 2016; Wood *et al.*, 2017). Previous studies reported using Illumina HiSeq and MiSeq, oil palm cultivation in a tropical forest ecosystem did not affect the bacterial diversity and ecosystem functioning (Lee-Cruz *et al.*, 2013; Tin *et al.*, 2018; Tripathi *et al.*, 2016). The predominant bacterial taxa in oil palm plantations are Proteobacteria, Actinobacteria, Acidobacteria, Bacteroidetes and Firmicutes (Rupert *et al.*, 2020; Tripathi *et al.*, 2016; Wood *et al.*, 2017). Based on the functional genes analysis using Illumina HiSeq, the most prevalent genes associated with DNA metabolism, RNA metabolism, protein metabolism, cell division and cell cycle are relatively more abundant in oil

palm plantations compared to forests and logged forests (Tripathi *et al.*, 2016). Furthermore, using amplicon and shotgun metagenomic sequencing, studies on the impact of selective logging and oil palm plantation revealed that structural and functional gene composition of the total soil microbial community is strongly influenced by the conversion of forests into agricultural land (Tripathi *et al.*, 2016). This study provides a preliminary framework and serves as a new metrics to facilitate selection of secondary forest land optimal for oil palm cultivation while preserving and conserving microbial diversity based on the knowledge of its soil microbial composition and genomic potential, as illustrated in Figure 4 (Ayob *et al.*, 2023). Using ITS amplicon sequencing, different mycelial fungal communities such as saprotrophic, mycorrhizal, and pathogenic fungi were characterised in oil palm plantations compared to selectively logged forests (Robinson *et al.*, 2020). The composition and function of certain bacterial and fungal communities in cultivated soils vary according to nutrient regimes and prevailing plant communities (Sun *et al.*, 2014).

CONCLUSION

The development of molecular tools and technologies have increased our knowledge on microbial diversity and function in oil palm plantation compared to traditional methods. Despite the challenges of the developed methods, culture-dependent methods are still employed for pure isolates and integrated taxonomy and the consolidated species concept, which uses both morphology and DNA sequence data, are now broadly accepted by modern taxonomists. With the advancement of molecular technology, NGS has flourished in the identification and characterisation of microbial communities and functional studies on DNA from environmental samples. The use of this technique provides a new way of assessing soil microbial diversity and ultimately, a complete understanding of the potential impacts of environmental processes and human activities on soil microorganisms to improve soil fertility and promote soil quality. This would help to address ecological issues related to ecosystem services in agricultural systems, especially in oil palm plantations. In addition, a clear understanding on microbial data is crucial to develop robust solutions in managing oil palm plantation for various aspects of soil health, ecosystem restoration, the use of microbial resources, and climate change. Proper oil palm management practices could be adopted and implemented by farmers and stakeholders which will allow sustainable oil palm production and conserve ecosystem functions. Future studies of soil

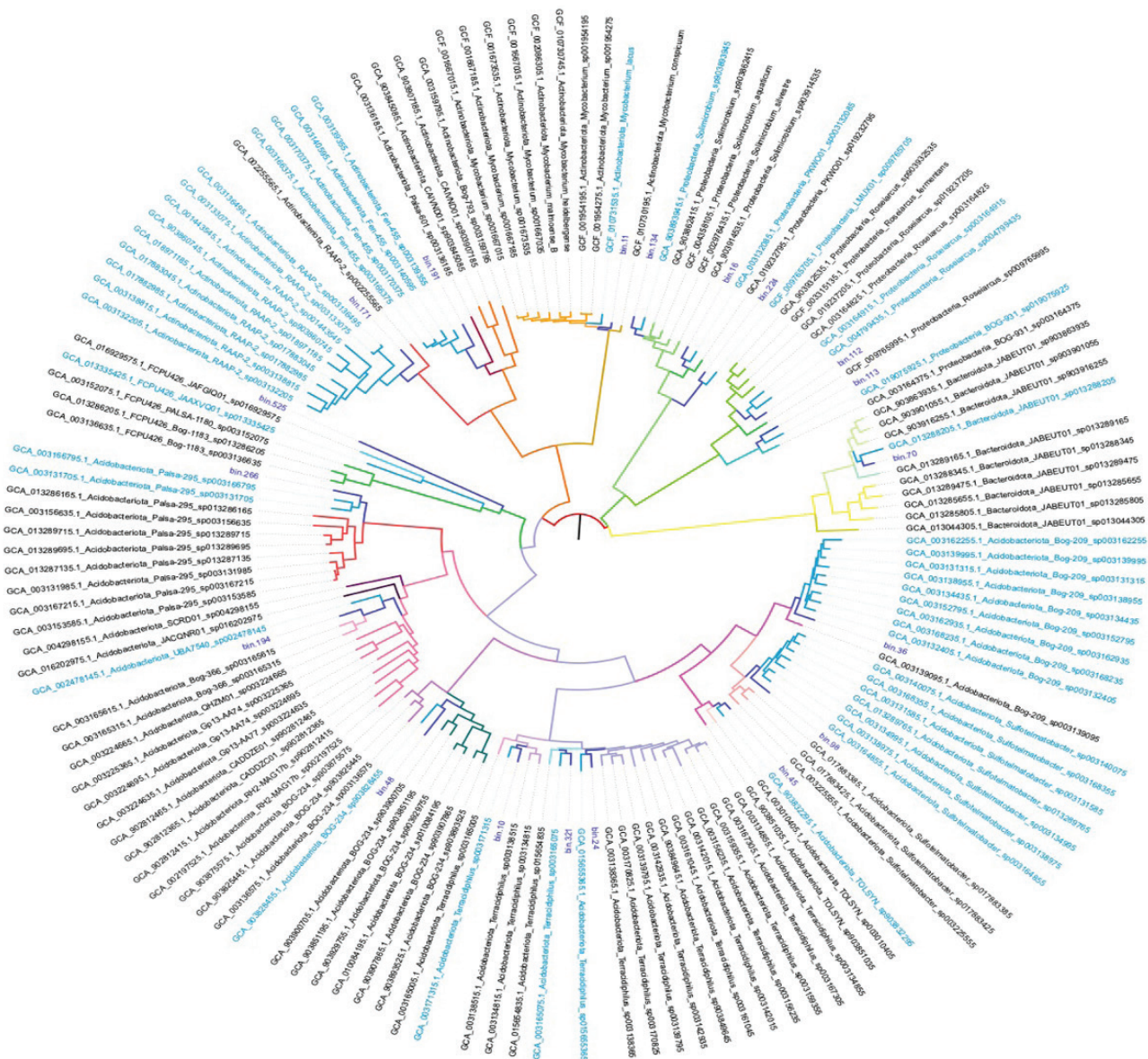


Figure 4. Maximum likelihood tree showing the phylogenomic placement of bacterial metagenome-assembled genomes (MAGs) against closely related representative genome from the Genome Taxonomy Database (GTDB) at logged forest soil using Illumina NovaSeq 6000. Branches were coloured based on the phylum and genus classification. Taxa forming immediate monophyletic group with the MAGs were coloured light blue while the MAGs were coloured blue.

microbial communities must rely on a combination of culture-dependent and culture-independent approaches. This will allow the development of an efficient monitoring tool and assessment of the long-term implications of such changes for agricultural sustainability, and as a bio-indicator to be included in the management and other decision making in the oil palm industry.

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