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Editor-in-Chief
Journal of Oil Palm Research
Malaysian Palm Oil Board
6 Persiaran Institusi, Bandar Baru Bangi
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Tel: *603-8769 4400*
E-mail: *pub@mpob.gov.my*
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OIL PALM ECONOMIC PERFORMANCE IN MALAYSIA AND R&D PROGRESS IN 2021

GHULAM KADIR AHMAD PARVEEZ[1]*; NUR NADIA KAMIL[1]; NORLIYANA ZIN ZAWAWI[1]; MEILINA ONG-ABDULLAH[1]; RAHMAHWATI RASUDDIN[1]; SOH KHEANG LOH[1]; KANGA RANI SELVADURAY[1]; SENG SOI HOONG[1] and ZAINAB IDRIS[1]

ABSTRACT

The palm oil industry fared better in 2021 compared to 2020, despite lower crude palm oil (CPO) production due to labour shortage and a restricted supply of CPO during the COVID-19 pandemic. As the industry recovers, research and development (R&D) activities remain dedicated towards ensuring the industry is sustainable and competitive. In the upstream sector, efforts continue to be focused in increasing the CPO yield per hectare through precision agriculture, advanced genomic technologies and improved breeding programmes, control of pest and diseases, as well as farm mechanisation. In the midstream sector, there were some improvements in mill productivity, that reduce the environmental impact of the milling operations. Intensification of R&D related to palm-based biomass has the potential to contribute to higher income for the industry. In the downstream sector, food safety and the nutrition-rich value of palm oil offer the best quality for this versatile and productive oil crop, to the world. Additionally, non-food products such as biofuels, biopolymers and bio-lubricants are also gaining research traction due to global movement towards a circular economy and sustainability.

Keywords: bioenergy, biomass, food safety and nutrition, oleochemicals, sustainability.

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INTRODUCTION

Oil palm is the most productive vegetable oil crop in the world, with a potential yield of 4 to 5 t of CPO ha^{-1} yr^{-1} (Hashim *et al*., 2010). Being the most efficient oil crop, palm oil production from major producing countries constituted about 31.6% (76.39 million tonnes) of global total oil and fats production (241.36 million tonnes) for 2021 (Oil World, 2021). This massive oil production comes from a meagre 6% of global agricultural land devoted to oil crops (Our World in Data, 2021), which further strengthens palm oil's sustainability in terms of land usage.

On the domestic front, Malaysia produced about 18 million tonnes of CPO in 2021, which accounted for about 8.5% of the global oils and fats production. The export of palm oil and oil palm products generated RM108.52 billion in revenue for Malaysia in 2021 (MPOB, 2022a). It is noteworthy that palm oil is one of the major contributors to the nation's export revenue, aside from electrical and electronic products as well as petroleum products (MATRADE, 2022). Furthermore, the Malaysian palm oil industry provides more than half a million employees and supports the livelihood of an estimated one million people (MPIC, 2018). Thus, the palm oil industry is vital for the economy and well-being of the country.

The year 2021 was indeed challenging for the palm oil industry, as the country was recovering from the economic downturn caused by the COVID-19 pandemic. However, the emergence of new COVID-19 variants has somewhat decelerated the progress of national economic recovery, as priority was placed on the nationwide vaccination programme. The situation was further exacerbated by the closure of international borders and the departure of foreign labour resulting in an acute

[1] Malaysian Palm Oil Board,
6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.

* *Corresponding author e-mail: parveez@mpob.gov.my*

shortage of workers in oil palm plantations. This led to an acute harvesting problem of oil palm fruits, which triggered its restricted supply in the market. Consequently, the prices of CPO have reached historical highs that subsequently contributed to higher export revenue for the industry. Nonetheless, the labour shortage offsets the gain in export revenue as the Malaysian palm oil industry lost billions of ringgits due to unharvested ripe fresh fruit bunches.

Ideally, the high price of CPO should be driven by strong demand for palm oil and oil palm products, together with high CPO production and high sales volume. Such a situation can only be realised via continuous R&D efforts across the palm oil supply chain covering upstream, midstream and downstream sectors, in tandem with the implementation of appropriate policies that make palm oil sustainable, safe and competitive. As such, R&D goals must be geared towards improving oil palm yield per hectare through precision agriculture, adoption of advanced biotechnology and breeding approaches to produce high yielding planting materials. These planting materials are tailored to be resistant to pests and diseases, resilient to climate change and facilitate farm mechanisation operations to overcome productivity issues caused by harvester shortage. Moreover, about 94.4% of the oil palm planted area has been Malaysian Sustainable Palm Oil (MSPO) certified, since the inception of the certification scheme in 2017. The MSPO certification complements the existing international certifications and ensures Malaysia meets the stringent market demands for sustainable palm oil.

The food safety and nutritional aspects of palm oil remain important and relevant to ensure its highest standard and quality for global food consumption. Additionally, R&D efforts enhance the circular economy of the industry through the utilisation of oil palm biomass and by-products as feedstock for various industrial applications. On top of that, enhancing the utilisation of palm oil mill effluent (POME) for biogas power generation is also desirable to boost the sustainability and environmental friendliness of the palm oil industry.

Moving forward, new palm oil usage in the food and non-food sectors is crucial to expand the market share and create new market segments in the global oils and fats economy, to maintain a healthy profit margin for the industry. Some examples of new and innovative applications are those associated with palm phytonutrients, cocoa butter alternative, bio-jet fuel, bio-polyol and polyurethane, bio-lubricants and personal care products.

This article provides valuable insights on the performance of the Malaysian palm oil industry in 2021 and reviews significant research advancements and innovative solutions across the whole supply chain of the palm oil industry. It also attempts to deliberate on strategies and future directions that the industry could embark on, to further enhance its competitiveness towards achieving a sustainable palm oil industry.

PERFORMANCE OF MALAYSIAN OIL PALM INDUSTRY

The year 2021 was deemed challenging as the COVID-19 pandemic continues to be threatening and hampering economic recovery efforts. Although 2021 was set for economic revival, the emergence of new variants of COVID-19 had somewhat decelerated the efforts. The economic activities were operated under great uncertainty and heavy pressure. In Malaysia, apart from rolling-out the vaccination programme, stringent border controls as well as strictly localised lockdowns were the critical strategies to contain the spread of the virus. These strategies, however, also affected the plantation sectors which rely heavily on foreign workers. The closures of international borders and the prolonged suspension of foreign worker intake affected oil palm harvesting activities and consequently have taken a toll on palm oil production. The significant decline in production has resulted in a much-reduced stockpile, hence limiting the capacity of Malaysia to export and ultimately pushing the price of the CPO to an all-time high, peaking several times in 2021.

Planted Area

The containment measures implemented to curb the spread of the COVID-19 pandemic had affected palm oil production in Malaysia. The series of local lockdowns had also slowed down replanting activities in oil palm plantations. In 2021, the total oil palm planted area had reduced by 2.2% to 5.74 million hectares from 5.87 million hectares recorded in 2020. At the regional level, planted area in Peninsular Malaysia and Sabah had declined by 4.7% and 1.3% against that of the previous year to 2.61 million hectares and 1.52 million hectares, respectively. Meanwhile in Sarawak, the oil palm planted area had increased by 1.4% to 1.61 million hectares (*Table 1*).

A similar trend was observed for the oil palm matured area. In totality, the oil palm matured area accounted for 5.14 million hectares or 89.7% of the total oil palm planted area, which was 1.7% lower than that of the previous year. The total matured area in Peninsular Malaysia was 2.36 million hectares, followed by Sarawak at 1.45 million hectares and Sabah at 1.33 million hectares. In terms

of ownership, 73.2% of the planted area was owned by private and government/state agency estates, 15.1% by the independent smallholders and 11.7% by the organised smallholders (*Figure 1*).

Status of Mills and Plants

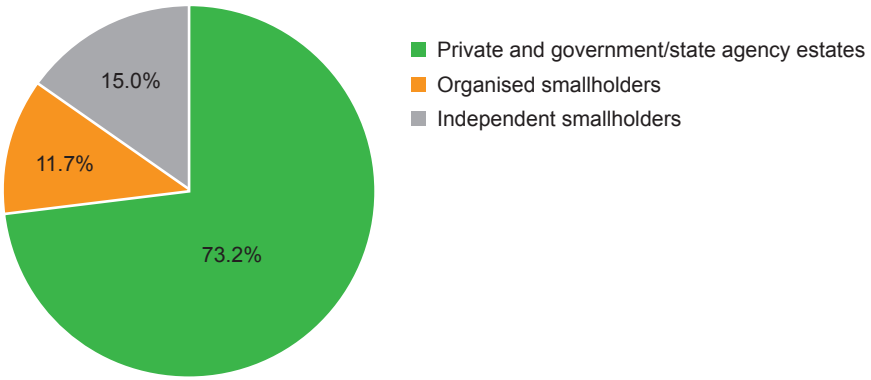
A total of 451 palm oil mills in Malaysia was in operation in 2021, with a combined annual processing capacity of 115.87 million tonnes of fresh fruit bunch (FFB), of which 52.50% of palm oil mills are located in Peninsular Malaysia with a total processing capacity of 57.50 million tonnes. The year 2021 had witnessed a decline in milling capacity utilisation rate by 5.70% to 77.56%, when compared to 82.26% recorded in the previous year, due to the lower FFB processed by palm oil mills (MPOB, 2022a). In the refining sector, a total of 49 palm oil refineries are in operation, with a total processing capacity of 25.76 million tonnes of CPO and crude palm kernel oil (CPKO). There are currently 33 refineries located in Peninsular Malaysia, with a total processing capacity of 14.28 million tonnes. The refining capacity utilisation rate for 2021 had reduced by 13.90% to 56.85% from 66.02% in the previous year, mainly due to the combination of lower CPO and CPKO processed, lower CPO production and higher CPO export (MPOB, 2022a).

Meanwhile, 43 palm kernel crushers were in operation with a total processing capacity of 7.42 million tonnes of palm kernel. There are 26 palm kernel crushers (60.5%) located in Peninsular Malaysia, with a total processing capacity of 4.59 million tonnes. The palm kernel crushing capacity utilisation rate had declined by 2.70% to 62.74% from 64.48% in 2020 due to a reduction in processed palm kernel and a lower supply of palm kernel, which arose from lower FFB production (MPOB, 2022a). A total of 19 oleochemical plants are in operation, with processing capacities of 2.67 million tonnes. These oleochemical plants processed a total of 2.25 million tonnes of palm oil products, which had declined by 9.7% when compared to 2020. The capacity utilisation rate of the oleochemical sector had also declined from 94.6% in 2020 to 84.1% in 2021, due to thelower volume of palm oil and palm kernel oil (PKO) processed (MPOB, 2022b). For biodiesel, there are currently 18 biodiesel plants in operation, with production capacities of 2.33 million tonnes. In terms of output, the total production of biodiesel was estimated at 0.92 million tonnes, an increase of 1.1% from 0.91 million tonnes in 2020 (Oil World, 2022). These oleochemical and biodiesel plants are mainly located in Selangor and Johor with eight and seven oleochemical plants, respectively, and six biodiesel plants in Selangor and Johor, respectively.

TABLE 1. MALAYSIAN OIL PALM AREA AS IN DECEMBER (ha)

	Planted area			Matured area		
	2021	2020	Difference (%)	2021	2020	Difference (%)
Peninsular Malaysia	2 607 847	2 737 723	(4.7)	2 363 870	2 455 535	(3.7)
Sabah	1 523 624	1 543 054	(1.3)	1 331 981	1 344 608	(0.9)
Sarawak	1 606 261	1 584 520	1.4	1 448 329	1 431 600	1.2
Malaysia	5 737 731	5 865 297	(2.2)	5 144 180	5 231 743	(1.7)

Source: MPOB (2022a).



Source: MPOB (2022a).

Figure 1. Oil palm planted area by category in 2021.

CPO Production

The average FFB yield for Malaysian oil palm estates in 2021 had declined to 15.47 t ha^{-1}, which was 7.5% lower compared to that of the previous year. The highest decline was recorded in Peninsular Malaysia, followed by Sarawak and Sabah, with a year-on-year percentage decline of 8.60%, 7.00% and 6.40% each (*Table 2*). Despite the decline in FFB yield, the oil extraction rate (OER) for CPO had increased slightly by 0.50%, and increased year-on-year to 20.01% from 19.92% in 2020. However, the slight increase in the OER was still unable to offset the decline in the average FFB yield of Malaysian estates, hence had brought CPO production much lower than that in 2020.

CPO production had dropped by 5.4% year-on-year to 18.12 million tonnes, the lowest level since the occurrence of the *El-Nino* event in 2016. This decline was mainly attributable to the disruption in labour supply in oil palm plantations, arising from the freeze on foreign workers due to COVID-19 containment measures. The number of foreign workers that were employed in the oil palm plantation sector had reduced consecutively in 2020 and 2021 by 2.3% and 8.7%, respectively (MPOB, 2019; MPOB, 2020; MPOB, 2021). Zooming into the regional performance, the CPO production in Peninsular Malaysia, Sabah and Sarawak were at 9.85 million tonnes, 4.36 million tonnes and 3.91 million tonnes, respectively (*Table 3*).

Palm Oil Exports and Imports

To support the increasing demand for the domestic processing sector, Malaysia imported 1.50 million tonnes of palm oil and other palm-based products (POPP) in 2021, 16.7% higher than that in 2020 (*Table 4*). The import of palm oil accounted for 78.3% of the total POPP imports with a volume of 1.18 million tonnes. This was 24.3% higher than that recorded in 2020. Almost all palm oil imports in Malaysia were sourced from Indonesia, wherein the amount was 1.14 t or 96.6% of total palm oil imports.

Regarding exports, the lower production of CPO in 2021 had limited the capacity of Malaysia to supply palm oil to the global market. The total export of POPP was estimated at 25.44 million tonnes, 4.4% lower than the previous year. The decline in the exports of POPP was mainly attributed to the drop in exports of palm oil, PKO and palm kernel cake by 8.5%, 0.7% and 11.1%, respectively (*Table 5*).

TABLE 2. AVERAGE FFB YIELD FOR MALAYSIAN OIL PALM ESTATES (t ha^{-1})

	2021	2020	Difference	
			Volume	%
Peninsular Malaysia	16.24	17.76	-1.52	-8.6
Sabah	15.77	16.84	-1.07	-6.4
Sarawak	13.94	14.99	-1.05	-7.0
Malaysia	15.47	16.73	-1.26	-7.5

Source: MPOB (2022a).

TABLE 3. MALAYSIAN CRUDE PALM OIL (CPO) PRODUCTION (t)

	2021	2020	Difference	
			Volume	%
Peninsular Malaysia	9 847 022	10 438 899	(591 877)	(5.7)
Sabah	4 362 698	4 647 375	(284 677)	(6.1)
Sarawak	3 907 820	4 054 339	(146 519)	(3.6)
Malaysia	18 117 540	19 140 613	(1 023 073)	(5.3)

Source: MPOB (2022a).

TABLE 4. MALAYSIAN IMPORTS OF PALM OIL AND OIL PALM PRODUCTS (t)

	2021	2020	Difference	
			Volume	%
Palm oil	1 177 251	946 917	230 335	24.3
Palm kernel oil	273 691	281 514	(7 823)	(2.8)
Palm kernel	52 889	59 854	(6 965)	(11.6)
Total	1 503 831	1 288 285	(215 546)	16.7

Source: MPOB (2022a).

TABLE 5. MALAYSIAN EXPORTS OF PALM OIL AND OIL PALM PRODUCTS

	Volume (t)			Value (RM million)		
	2021	2020	Difference (%)	2021	2020	Difference (%)
Palm oil	14 835 115	16 214 373	-8.5	64 615	45 647	41.6
Palm kernel oil	1 131 771	1 140 204	-0.7	6 668	4 152	60.6
Palm kernel cake	2 248 391	2 529 444	-11.1	1 394	1 295	7.7
Palm-based oleochemicals	4 760 115	4 423 971	7.6	26 803	16 501	62.4
Other palm-based products	2 466 031	2 302 922	7.1	9 035	5 738	57.5
Total	25 441 423	26 610 914	-4.4	108 516	73 332	48.0

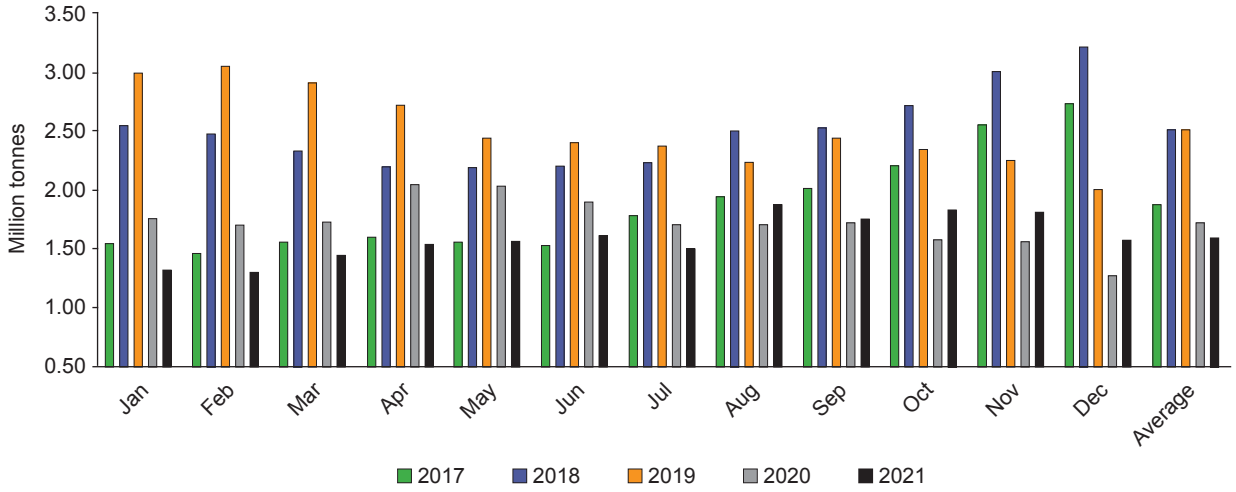


Figure 2. Palm oil monthly closing stocks and then the average stocks level for each year.

Palm oil accounted for 64.1% of the total exports of POPP. The decline in palm oil exports was due to the weaker demand from major importing countries such as China and the European Union (EU). Exports of palm oil to China have decreased by 31.4% year-on-year to 1.87 million tonnes. The shift in the sourcing countries for palm oil from Malaysia to Indonesia explained the significant decline in the exports of Malaysian palm oil to China. In addition, the higher soyabean imports from the USA have also influenced the intake of Malaysian palm oil by China. Exports of Malaysian palm oil to the EU have decreased by 15.4% year-on-year to 1.64 million tonnes because it was replaced by higher imports of soyabean from Brazil. Unlike China and the EU, palm oil exports to India have surged by 31.3% year-on-year to 3.60 million tonnes. This significant increase was due to low palm oil uptake from Indonesia in view of the higher CPO export tax imposed by Indonesia. The sharp increase in palm oil export to India has commanded India to be the largest palm oil export market for Malaysia.

Despite the low export volume, total export revenue of POPP recorded by the Department of Statistics Malaysia had surged by 48.0% to RM108.52 billion due to higher export prices of palm oil and other oil palm products (MPOB, 2022). Export revenue of palm oil and PKO had surged by 41.6% and 60.6% to RM64.62 billion and RM6.67 billion, respectively. A significant growth in export revenue was also recorded for other products such as palm kernel cake, palm-based oleochemicals and other palm-based products, with the year-on-year growth of 7.7%, 62.4% and 57.5%, respectively.

Closing Stock

The reduction in CPO production in 2021 had greatly affected the Malaysian palm oil industry. It not only had limited palm oil export capacity but also put significant pressure on the national stocks level. The monthly closing palm oil stocks hit below 1.50 million tonnes more than twice in 2021, which has never happened in the previous five years. This had brought the monthly average closing stocks of palm oil to 1.60 million tonnes, the lowest level since 2017 (*Figure 2*). The tight supply of palm oil stocks had pushed CPO price to a record high, breaking the RM5000 t^{-1} level.

Price

The supply-push factor drives the prices of all major oil palm products to be traded higher in 2021 compared to 2020 (*Table 6*). The local CPO price grew by 64.1% year-on-year to RM4407.00 t^{-1}

against RM2685.50 t^{-1} in 2020. The highest monthly price was recorded in November 2021 at RM5341.00 t^{-1}. Apart from the lower-than-expected decline in production, higher prices of soybean oil in the world market have also supported the increase in the CPO price. The movement in palm oil prices is influenced by the fluctuations in soybean oil prices as both oils are competing for a share in the global vegetable oils market. In addition, the firmer Brent crude oil prices during that year had made palm biodiesel more attractive, hence helping to support the rise in CPO price.

Along with the increase in CPO price, export prices of major processed palm oil products namely refined, bleached and deodourised (RBD) palm oil, RBD palm olein and RBD palm stearin had also surged by 70.0%, 67.5% and 64.2% to RM4748.50 t^{-1}, RM4764.50 t^{-1} and RM4598.00 t^{-1}, respectively while palm fatty acid distillate (PFAD) price rose by 66.3% to RM4233.00 t^{-1}. In the lauric market, the price of palm kernel had increased by 81.0% to RM2773.00 t^{-1} from RM1532.00 t^{-1} in 2020. This was mainly due to the higher domestic price of CPKO, which was improved by 74.8% to RM5674.50 t^{-1} compared to RM3247.00 t^{-1} in 2020. The higher CPKO prices were in tandem with the increase in lauric oil prices namely, PKO and coconut oil. PKO prices in the global market increased by USD691.00 or 83.7% year-on-year to USD1517.00 t^{-1} and the coconut oil price increased by USD603.00 or 59.5% year-on-year to USD1617.00 t^{-1}. Conforming to the CPO and palm kernel prices hike, FFB price at the mill gate had surged by 70.2% to RM955.00 t^{-1} against RM561.00 t^{-1} in 2020.

R&D FOCUS AREAS IN 2021

Precision Agriculture using Digital Technologies in Oil Palm Plantation

Since the first commercial oil palm estate was established in 1917, the palm oil industry in Malaysia has grown by leaps and bounds over the years to meet the ever-increasing global demand for food and non-food products (RSPO, 2015). The palm oil industry has generated many opportunities and societal benefits for the rural communities and has become an important contributor to the economies of Malaysia and Indonesia.

As oil palm cultivation needs continuous improvement to meet increasing demand, calls for a sustainable supply system have intensified globally. To achieve a balance between economic growth and environmental sustainability, intervention policies should address the concerns of deforestation through the conversion of degraded secondary forests and replacing other non-economically viable agriculture crops with oil palm (MohdAzlan *et al.*, 2021). The potential values of forest fragments and wildlife-friendly practices in oil palm landscapes and their roles in conservation in Malaysia need to be carefully evaluated. Agroforestry options such as mixed-species tree planting and natural regeneration in oil palm plantations may help alleviate the negative effects of forest biodiversity loss and to safeguard ecosystem functions (Donfack *et al.*, 2021).

Many studies have been carried out to monitor changes in ecology and biodiversity throughout the oil palm development processes so that the effects of land conversion can be minimised and managed. In 2021, most biodiversity studies were done on tropical peatlands, specifically in Sarawak. Amit *et al.* (2021) concluded that bird species diversity, abundance and their feeding guild can be improved by letting ground layer vegetation grow naturally and maintaining the water quality of the drainage system in the early stage of oil palm development to attract birds which prefer this habitat. Ayob *et al*. (2021) recovered 227 bacterial isolates belonging to four major phyla (22 genera) from culture-dependent and -independent approaches in oil palms planted on tropical peatland.

Using the denaturing gradient gel electrophoresis method, Wong *et al.*, (2021) reported that the soil fungal composition and diversity in oil palm plantations were significantly different

TABLE 6. MALAYSIAN PRICES OF OIL PALM PRODUCTS (RM t^{-1})

	2021	2020	Difference	
			RM	%
CPO (local delivered)	4 407.00	2 685.50	1 721.50	64.1
RBD palm oil (FOB)	4 748.50	2 794.00	1 954.50	70.0
RBD palm olein (FOB)	4 764.50	2 844.00	1 920.50	67.5
RBD palm stearin (FOB)	4 598.00	2 801.00	1 797.00	64.2
PFAD (FOB)	4 233.00	2 546.00		66.3
Palm kernel (ex-mill)	2 773.00	1 532.00	1 241.00	81.0
CPKO (local delivered)	5 674.50	3 247.00	2 427.50	74.8
FFB (mill gate)	955.00	561.00	394.00	70.2

Note: FOB -

Source: MPOB (2022a).

compared to undisturbed secondary forest and disturbed secondary forest at Sungai Asap, Sarawak. Uke *et al.* (2021) found an increase in the abundance of microorganisms involved in lignocellulose decomposition, due to unregulated disposal of oil palm trunk fibre into plantation areas. From 62 canopy ant species belonging to six subfamilies found in Central Borneo, Indonesia, Rizali *et al.* (2021) inferred that the occurrence of natural habitats helps shape similar ant community in oil palm plantations, possibly via inhibiting the abundance of invasive species.

Land use change from oil palm expansion has accelerated in the last few decades, inducing significant ecological, hydrological and atmospheric effects. In 2021, many studies provided information on the impacts of palm oil trade and its expansion on socio-economic and ecosystem (Ayompe *et al.,* 2021; Jaroenkietkajorn *et al.,* 2021; Krishna *et al.,* 2021), as well as a map of oil palm cultivated areas in Malaysia and Indonesia (Tapia *et al.,* 2021). The outcome of these studies could lead to the development of strategies to provide a sustainable oil palm plantations ecosystem and to meet Sustainable Development Goals such as ensuring healthy living and promoting well-being as well as responsible consumption and production.

Research addressing agronomic and environmental issues in oil palm plantations aimed at increasing crop yield and minimising environmental impacts were also reported. Norizan *et al.* (2021) estimated oil palm water demand by using the FAO-CROPWAT model to manage irrigation plans prior to the project implementation because such site-specific implementation is risky and costly. Two studies focused on the effects of water deficit on the physiological state of young oil palm (Filho *et al.,* 2021) and seedlings of two different genotypes of *Elaeis guineensis* and four interspecific (*E. oleifera* × *E. guineensis*) hybrids (Tezara *et al.,* 2021) were also conducted. Rudolf *et al.* (2021) concluded that empty fruit bunch (EFB) mulching increases the sustainability of oil palm smallholders, provided the supply constraints can be resolved.

In India, Behera *et al.* (2021) have established an efficient soil nutrient (K, Ca, Mg) management system through soil and leaf nutrients stoichiometry. Looking into soil health and sustainable production, Mahmud *et al.* (2021) have described the potential use of EFB biomass as biofertiliser and the roles of growth-promoting microbes for plant growth and development. Several studies have been reported on carbon dioxide emissions and the value of carbon stocks from different oil palm ecosystems, *i.e.*, the replanting phase (Kusumawati *et al.*, 2021), peat soils in Sarawak (Mos *et al.,* 2021) and in Sumatra (Rahman *et al.,* 2021). Proper interpretation of data published is important for site specific management.

Similar to 2020, there were noticeably many research publications addressing issues on pests and diseases in oil palm cultivation. Siddiqui *et al.* (2021) have critically reviewed the progress made in Basal Stem Rot (BSR) development and management in oil palm and suggested that all control methods should be re-evaluated and improved to prevent, treat and ultimately control the threatening effects of BSR. The yield losses due to BSR disease was estimated using Bayesian Model Averaging, which indicated that the most important predictor was the planting preparation technique, followed by disease progression, disease severity, number of infected neighbouring palms, and two interaction effects (Kamu *et al*., 2021). Fahrizal *et al.* (2021) explored the potential of *Syncephalastrum racemosum* and *Rhizopus arrhizus* isolated from oil palm trunks to produce chitosans known to inhibit the growth of *Ganoderma boninense,* the main causing factor of BSR. Studies were also conducted to investigate the effect of commonly used herbicides in oil palm plantations, as a predisposing factor to BSR disease development (Hussin, *et al.,* 2021).

Besides *Ganoderma*, the bunch moth is also a recurrent problem in the oil palm plantations. Ming *et al.* (2021) have established the Economic Injury Level (EIL), the percentage of fertile oil palm fruitlets and oil to bunch index at different infestation severity of the bunch moth, *Tirathaba mundella* Walker on oil palm (*Figure 3*). Sulaiman *et al.* (2021a) have reported the utilisation of light-trapping with fluorescent bulbs, which resulted in a significantly higher capture of *Tirathaba mundella*. It was also argued that the bunch moth could have developed resistance over time, due to the frequent exposure to *Bacillus thuringiensis*-based insecticide in the field, as well as irregular applications of insecticide to contain the situation (Khai *et al.,* 2021). Apart from that, the noxious weed growing in oil palm plantations, *Eleusine indica* (goose grass), was also reported to have developed resistance to glyphosate (Purba *et al*., 2021). Meanwhile, the population density of *Elaeidobius kamerunicus* was found to be affected by different soil types, *i.e.*, mineral and peat soils, and the availability of oil palm male inflorescences (Mohamad *et al.,* 2021).

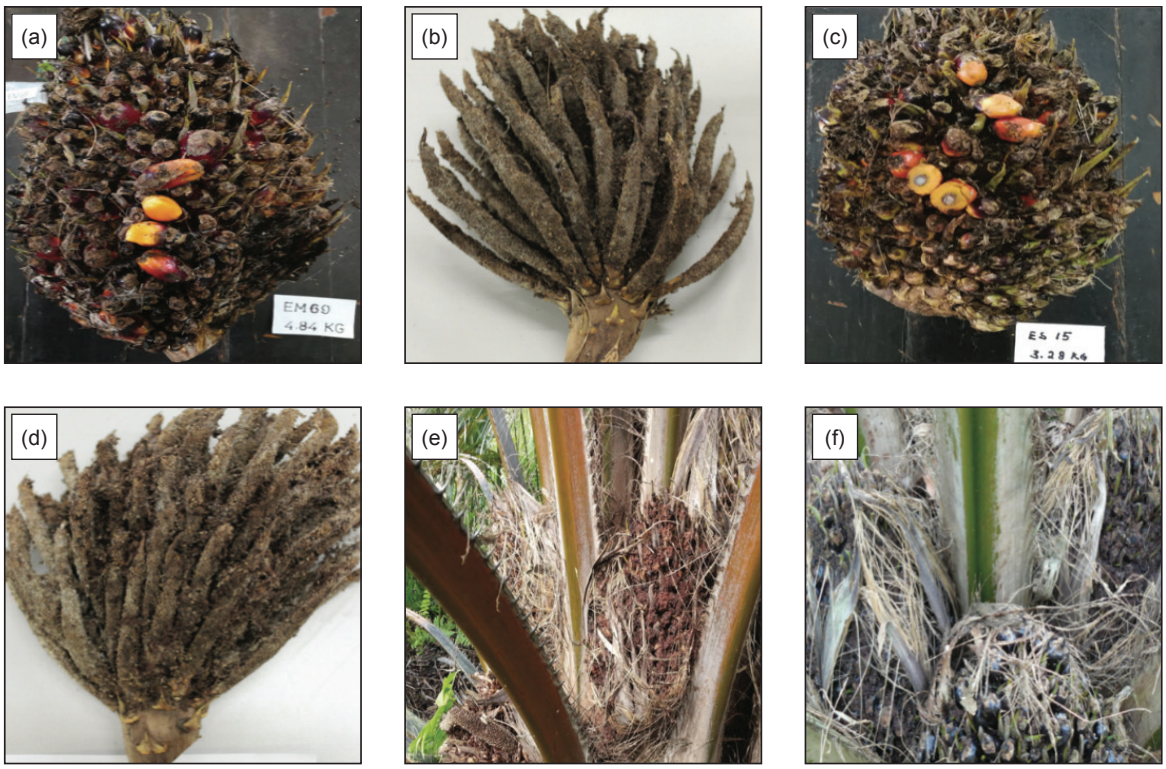
In the last decade, artificial intelligence, predictive analytics, the Internet of Things and other technologies, have emerged as essential tools for modern agriculture. These technologies help to strengthen precision agriculture to overcome challenges in the industry, especially for decision making, based on field spatial and temporal variability. Due to the high cost involved in adopting these technologies, it is therefore important to arrive at the right item, in the right place and at the right time.

Accurate mapping of oil palm is important for understanding its past and future impact on the environment. Rodriguez *et al*. (2021) estimated oil palm areas via a new, active deep learning method using images from Sentinel-2 satellite. He reported that there are more than 1.2 billion oil palms planted in Indonesia, covering more than 15.0 million hectares, while in Malaysia, it is only more than 0.5 billion oil palms, covering more than 5.0 million hectares. Oil palm mapping was also used to illustrate the geographical pattern of oil palm development in different parts of a country to calculate the associated reduction in other types of land uses. In Guatemala, Hervas (2021) concluded that oil palm cultivation has compromised local food systems in many of the poor rural regions where households rely mostly on locally produced and/or self-provisioned food.

Reliable and accurate predictions in oil palm production can provide the basis for making decisions with regard to budgeting, storage, distribution, and marketing. Digitisation of data collection processes in the fields will reduce dependency on labour and hence reduce the production cost. Sensors, cameras, drones, and other devices help to gather real time data, with devices that are on the ground continuously. Mohammad *et al.* (2021) managed to spatially visualise the nitrogen (N) status of immature oil palm area with an autopilot tractor-mounted active light sensor, while Zheng *et al.* (2021) observed oil palm growth using Unmanned Aerial Vehicle captured images.

Proper interpretation of yield maps for site-specific management can help to increase crop yield. For example, Martinez *et al.* (2021) evaluated yield variability using terrain algorithms on a digital elevation model, while Hilal *et al.* (2021) developed Artificial Neural Network and Non-linear Autoregressive Exogenous Neural Network models to predict FFB yields in Peninsular Malaysia. Suharjito *et al.* (2021) created a mobile application to classify the ripeness levels of FFB using a lightweight Convolutional Neural Network and Ahmad *et al.* (2021a) identified the spectral signature of the bagworm species of *Metisa plana* Walker initiated by using Visible/Near Infrared spectroscopy. Adoption of all geospatial technologies in oil palm plantations is critical to assist in the decision-making process and hence, leading to effective management.

While advancing through technological innovations, the more immediate solution currently implementable is mechanisation. Several studies are currently focusing on health issues of harvesters in oil palm plantations. In handling a harvesting tool



Source: Ming *et al.* (2021).

Figure 3. (a) Ripe oil palm fruit bunch with moderate oil palm bunch moth infestation. (b) Post-anthesis oil palm male inflorescence with moderate oil palm bunch moth infestation. (c) Ripe oil palm fruit bunch with a severe bunch moth infestation. (d) Post-anthesis oil palm male inflorescence with severe bunch moth infestation. (e) Oil palm fruit bunches with severe new infestation covered by reddish faeces. (f) Oil palm fruit bunches with severe old infestation covered by brownish-black faeces.

for FFB, some level of musculoskeletal disorders could be detrimental to workers, in particular the shoulders and trunk area, due to posture distortion and responses to muscle activity. During loading of FFB using spike, a force of 16.36 N would impart the left triceps of a worker (Mohamaddan *et al*., 2021). Adjustments in the design of the tool, task undertaken and working shifts may help in addressing this issue. On the other hand, an upper limb-assisted exoskeleton prototype has been found able to reduce up to 30.0%-50.0% of selected muscle activities of an oil palm harvester while handling a harvesting pole (Harith *et al*., 2021). Though encouraging, the design needs to be further optimised for better technology acceptance. In addition, a global positioning system-assisted mathematical model (Lim *et al*., 2021a) has been utilised to optimise harvesting routes of farmers and transporters by reducing up to 26.3% travelled distances, hence leading to savings of time and resources devoted to different harvestable plantation sites.

Integrative Science Driving Future Sustainability

Lately, the palm oil industry has been demonised rampantly. Food and hygiene products are brazenly carrying labels such as 'Proudly Palm Oil Free' (Divinechocolate, 2022) and "Always Palm Free" (Siliskisoaps, 2022) respectively, despite Indonesia and Malaysia, the major producing countries, striving for recognition as certified sustainable palm oil producers. This action simply negates the grandeur efforts by the palm oil industry to ensure sustainable practices are deeply entrenched, regardless of whether the produce is sourced from big plantations or smallholdings. The key contentious issue surrounding the palm oil industry is its impact on the environment, in particular, deforestation and loss of biodiversity (Vijay *et al*., 2016). Substitutes of palm oil, such as other vegetable oils, cannot replicate palm oil's versatility and productivity per land area while a synthetic replacement is still a work in progress (Parsons *et al*., 2020). As such, research on oil palm continues to move forward with sustainability, conservation of biodiversity, and food security as the common themes.

Advanced biotechnologies offer remarkable potential in crop improvement for oil palm. In most cases, these tools are adapted from model systems that are further optimised for use in oil palm. Yeap *et al.* (2021) recently reported their success in establishing an efficient Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated Protein 9 (Cas9) mutagenesis system for the oil palm. The key optimising step was in determining the most effective cleavage and delivery methods to ensure efficient gene editing. While the CRISPR/Cas9 system undergoes further improvements for use in oil palm, utilisation of model species continues to be an essential tool for research advancement. The CRISPR/Cas9 method was employed to knock-out the OsFAD2-1 gene in rice as a model system. The intention is to subsequently repeat this highly effective approach in knocking out the fatty acid desaturase genes in oil palm, to produce higher oleic acid in palm oil (Bahariah *et al*., 2021).

Another potential component, carotenoids, at higher concentrations, can substantially enhance the quality of palm oil. Wan Nur Syuhada *et al*. (2021) had molecularly characterised the *phytoene synthase* (*psy*) gene, responsible for the synthesis of carotenoid in oil palm. Another oil quality characteristic studied is vitamin E, of which Shahrul *et al*. (2021) observed a Single Nucleotide Polymorphism (SNP) conversion from TAAT to form the CAAT-box which could be the promoter activity enhancing factor leading to an increased total tocotrienol content. Apart from manipulating genes to improve the quality of the oil, research focus has also been on developing more robust and resilient oil palm planting materials that are tolerant to abiotic stress such as drought.

As oil palm is highly reliant on water availability, prolonged drought stress could result in severe yield loss (Rodrigues Neto *et al*., 2021). It is known that metabolites are the direct representation of plant phenotypes, therefore, their signatures would provide biological and chemical fingerprints of the reactivity of oil palms toward external stimuli (Shulaev *et al*., 2008). The high capacity in compound detection and identification as well as the availability of methods to link pathways have made metabolomics an ideal tool for such comparative study (Vargas *et al*., 2016). Using a web-based tool known as the MetaboAnalyst 4.0, coupled with robust analysis algorithms, Rodrigues Neto *et al*. (2021) was able to identify five key metabolic pathways affected by drought stress: starch and sucrose metabolism; glyoxylate and dicarboxylate metabolism; alanine, aspartate and glutamate metabolism; arginine and proline metabolism; and glycine, serine and threonine metabolism. Ishak *et al*. (2021) provided a comprehensive comparative survey of statistical tools that can be utilised. Ultimately, the knowledge of the affected pathways could be developed into biotechnological applications in the development of climate resilient genotypes, especially when integrated with other omics approaches.

Zhou and Yarra (2022) opted for a more targeted approach in which *bZIP* transcription factors were identified from the oil palm using a genome-wide approach. This class of transcription factors is known to be important in regulating various developmental and biological processes aside from being involved in stress responses in plants.

Through detailed characterisation of the *bZIP* transcription factors obtained from public oil palm genome databases, the authors identified several tissue-specific *EgbZIP*s as well as 11 *EgbZIP*s to be highly expressed under abiotic stresses such as cold, salinity and drought. By applying the same concept, the group expanded their research into the role of the auxin response factor (ARF) gene family (Jin *et al*., 2022). Similarly, in this case, they were able to confirm 19 *EgARF* is prominently involved when oil palm is subjected to abiotic stresses.

Research into developing both biotic and abiotic resilient planting materials has become more prominent in recent times, mainly driven by the threat of climate change. However, this strategy needs to be carried out in tandem with enhancement of yields. A review by Babu *et al*. (2021) appositely touched on the importance of leveraging a combination of factors, from the extensive use of genetic resources to the employment of various breeding methods coupled with the use of advanced omics and bioinformatics tools. This holistic approach ensures that all bases are covered in addressing oil palm crop improvement. The oil palm germplasm is an invaluable asset which is currently maintained *ex-situ* as a living collection in the field (Rajanaidu *et al*., 2017). This manner of conserving the genebank is necessary as the genetic materials are used for the evaluation of new and improved traits to select future breeding programmes.

However, this presents several challenges: the requirement of a large land mass, high maintenance cost, as well as their probable exposure to pests and diseases (Gan *et al*., 2021). In tackling these issues, Gan *et al*. (2021) introduced the use of molecular markers to determine the genetic diversity of the Nigerian-based germplasm paired with a robust statistical analysis method to assemble them into a reduced core set of palms with minimum redundancies while preserving its diversity. This approach is deemed most sustainable in the long term, in handling germplasms and MPOB is following suit in this endeavour (Myint *et al*., 2021).

Seyum *et al*. (2021) reiterated the importance of incorporating high-density molecular markers onto large mapping populations as well as exploring the latest genome mapping software *e.g*., Lep-MAP3 as a means to accelerate oil palm breeding while enhancing its economic products. In line with this, Zolkafli *et al*. (2021) reported the use of 4451 SNP and more than 600 Simple Sequence Repeat (SSR) markers which led to the revelation of several common quantitative trait loci (QTLs) associated with yield components for two advanced breeding populations, namely the P2 (Deli *dura* x Yangambi *pisifera*) and KULIM DxP (Deli *dura* x AVROS *pisifera*). Further dissection of these regions provided clues on candidate genes impacting yield which can further be developed into potential markers for oil palm genomic selection. Apart from focusing on the commercial hybrid populations, Tupaz-Vera *et al*. (2021), focused their efforts on improving the selection of the parental line, *dura*. Through progeny testing and meticulous phenotyping, they successfully selected elite dwarfed *dura* parents with high yields for their commercial cultivar development.

Nonetheless, the limited genetic diversity of existing *Elaeis guineensis* cultivars due to extensive breeding remains a contentious issue (Adon *et al*., 2021). In broadening the genetic base, wild populations of both *E. guineensis* and *E. oleifera* are leveraged. Genotyped wild *E. oleifera* as reported by Ithnin *et al*. (2021) presents an opportunity for SNPs linked to key agronomic traits such as yield and fatty acid composition to be utilised in future breeding programmes via marker-assisted selection and genetic modification.

Using molecular tools, Sarimana *et al*. (2021) not only studied the genetic diversity of the hybrid (DxP) oil palm populations but extended the use of the molecular information in the form of DNA fingerprints of the individual palms to track their parental origins. This becomes important in the case of fraudulent seed trade (Cheyns and Rafflegeau, 2005). The assimilation of genome technology into the oil palm seed supply has been an ongoing process since the discovery of the *SHELL* gene assay (*Figure 4*) (Singh *et al*., 2013) but became more critical when Ooi *et al*. (2016) established a non-*tenera* contamination rate of 10.9% in the supply chain. The issue at hand is not new as it has been highlighted by Parveez *et al*. (2020). One of the key reasons for the slow acceptance by the industry is the imminent increase in the cost of the planting materials. In view of this, Singh *et al*. (2021b) explored the potential of statistical seed testing in addition to the established method of leaf sampling at nurseries encompassing all the possible *SHELL* variants in commercial populations associated with the fruit form phenotype (Ooi *et al*., 2016).

This bunch-by-bunch destructive sampling strategy enables the culling of contaminated bunches before they get into the supply chain. A quality control tool powered by genome technology is in line with the industry's aspiration towards a more extensive adoption of technology as well as underscores the sustainable efforts invested by the palm oil industry. In a follow up article by Singh *et al*. (2021a), the *SHELL* gene testing innovation has been instrumental in exposing the 10.9% non-*tenera* contamination amongst seedlings (Ooi *et al*., 2016), a level which is twice the permissible rate of the Malaysian Standards MS157:2017 at 5.0%. The economic analysis further demonstrated an annual monetary gain of approximately RM2.6 billion yr^{-1} could potentially be realised if 100.0% *tenera* were planted (*Figure 4*).

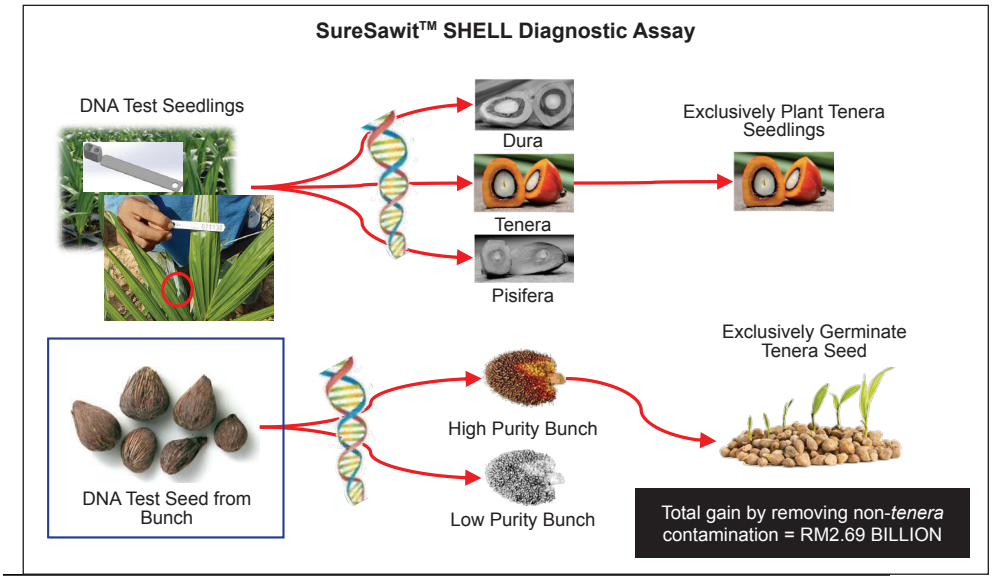


Figure 4. Authentication of tenera *planting materials via SureSawit™* SHELL *DNA testing of seedlings and seeds.*

In knowledge building, the primary input or building block of a system is the data. Oil palm research as with other crops has benefited from the advent of technologies as well as the publicly available deluge of information to progress research. Method development and protocol optimisation may be mundane but are crucial for research to advance to the more exciting phase of discovery. Azimi *et al*. (2021) demonstrated the tediousness of organellar related research, especially in dealing with the mitochondria. Similarly, Nagappan *et al*. (2021) experimented with several protocols to finally confirm the Boehm method to be the best suited for *Ganoderma zonatum* DNA extraction. Following suit, Apriyanto and Tambunan (2021) reported and publicly deposited the first draft genome of the oil palm pollinating weevil, *Elaeidobius kamerunicus*, a valuable resource to study the weevil in-depth. It is envisioned that this would pave the way for more weevil related research in the near future.

Kok *et al*. (2021) in proteomics, Sarpan *et al*. (2021) in epigenetics and Nadzirah *et al*. (2022) in transcriptomics, have through their research facilitated the enrichment of valuable data into the oil palm knowledge database. Trending now is the emphasis on applying robust data analytics to interpret research data (Sarker, 2021). Ooi *et al*. (2021) identified a total of 171 genes deemed able to potentially discriminate highly embryogenic ortets, with an upregulation observed amongst genes related to flowering time. Despite these findings, the authors opined that for practical use of these leaf expression biomarkers as a predictive tool, the integration of machine learning techniques is needed to further improve the sensitivity of the model.

Sustainable Development for Smallholders

The MSPO certification scheme serves as one of the platforms to address the concerns over issues related to environmental, social and economic impacts of the palm oil industry in Malaysia. Incomplete information and technical errors during the application for MSPO certification, illiteracy rate and level of education of the smallholder, accessibility of the holding, and the lack of competent attending officers, are factors that have influenced the certification process during the MSPO pre-audit activities (Yap *et al*., 2021c). The existing standards should be revised to expedite the MSPO certification process for independent smallholders (ISH) in Malaysia, and to support our commitment towards sustainability of the palm oil industry. The implementation of Good Agriculture Practice (GAP) is also considered as the baseline for the MSPO certification. Mansor *et al*. (2021) revealed that 58% of the ISH partially complied with GAP requirements. Whereas, only 26% of ISH fulfilled the requirements, and thus, were eligible for the GAP certification. Two factors that significantly influenced the GAP compliance among the ISH were the respondents' education level and the age of oil palm plantation. The results of this study indicated that fertiliser application and record-keeping adopted by the ISH had significantly affected their compliance level in MPOB GAP Certification.

For the recognition of MSPO in the international market, the MSPO standard was approved as a tool for sourcing code by the Tokyo 2020 Olympics and Paralympic Games Organising Committee in June 2018. The first shipment of MSPO certified palm

oil to Japan had taken place in September 2019. Furthermore, a memorandum of understanding was signed in 2019 between Malaysia Palm Oil Certification Council and China Green Food Development Centre to create acceptance of MSPO certified palm oil in China. Moreover, a collaboration between Malaysia with Solvent Extractors' Association of India resulted in the adoption of MSPO principle into Indian Palm Oil Sustainability Framework. Finally, the signing of a Letter of Intent in November 2019 had given the opportunity for Malaysia to work with The Netherlands on the National Initiative on Sustainable and Climate Smart Oil Palm Smallholder programme which support the implementation of MSPO certification scheme among independent smallholders in Malaysia (Rahimi, 2021).

An extension officer plays a key role in the dissemination of oil palm technologies to the ISH. The study done by Nur *et al*. (2021) revealed that ISH has a positive perception and attitude towards extension services, contributing to a high acceptance level of extension service activities by extension groups among ISH. Despite the positive results reported, improvements especially relating to the lowest mean score of perception and attitude towards the Q&A and discussion sessions as well as extension officers responses are much needed. It is also suggested that extension officers be encouraged to use a variety of new teaching methods to ensure active interactions between smallholders and extension officers. Furthermore, extension officers must improve their skills and knowledge in extension services by participating in various courses and programmes. The Sustainable Oil Palm Growers Cooperative (KPSM) in Malaysia is responsible to boost the income of ISH whilst creating job opportunities. However, it seemed that members' participation could be affected more by the good governance factor as demonstrated by the cooperative, rather than the role of the extension officer (Ainul *et al*., 2021b).

Labour shortage has also impacted yields among the ISH, albeit at a smaller scale as compared to the plantations. According to Nazirah *et al*. (2021), the majority of oil palm ISH were not entirely reliant on foreign workers to carry out activities in their smallholdings. The study provide a insight which showed that ISH hired 21.8% of foreign workers for harvesting, 19.8% for weed control, and 16.8% for fertiliser application. These findings provided justification for the government to support and formulate relevant policies to meet the basic needs of the ISH. A survey by Ainul *et al*. (2021a) revealed six significant factors affecting FFB yields of the ISH in Sabah: the level of education, monthly household income, farm management status, weeding, pests and nutrient deficiencies and agricultural input costs. These findings are important as they can be used as guidelines by the relevant parties to implement strategies to improve the FFB yield of ISH.

The main purpose of livestock or crop integration with oil palm is to diversify the source of ISH income through optimum utilisation of land and natural resources available in the oil palm planted areas. Sohimi *et al*. (2021) revealed that a total of 67.7% of respondents who participated in the Crop Integration Scheme implemented by MPOB from 2016 to 2017, chose to integrate banana with oil palm, followed by pineapple and papaya. They prefer to sell the produce, instead of for their consumption. Overall, the income derived from this scheme was estimated at RM200 to RM600 ha^{-1} $month^{-1}$, depending on the types of crops. In another study, Shafirul *et al*. (2021) revealed that respondents who participated in the livestock integration scheme could end up earning additional income or *vice versa*. Respondents who failed to manage their livestock were faced with problems of high mortality rate and high operational costs. However, with good management practice, costs incurred in integration could be reduced and coupled with the sale of their livestock at market price, a positive net income can be achieved. Therefore, there is a need for renewed efforts in driving livestock or crop integration as the concept is clearly beneficial to smallholders.

Biomass and Bioenergy Innovations

The palm oil industry generates plentiful biomass resources and by-products for value addition towards the creation of a circular economy. Particularly, in the palm oil milling process, by-products such as palm oil fuel ash (POFA), palm oil clinker (POC) and residual oil of palm oil mill effluent (ROPOME) have been actively exploited as seen with their increasing research activities spanning from fundamental, applied to simulation work. Efforts have also been made to improve oil and biomass production in palm oil mills by minimising water consumption during oil palm sterilisation via integrating the boiler and the steriliser into a combined unit. This is achieved by omitting piping and continuous heating. An optimal sterilisation condition of 2.5 bars and 60 min provides good fruit-bunch separation with acceptable oil quality, and four times lesser water consumption than the conventional method (Wae-hayee *et al*., 2022). Instead of sterilising whole palm fruits, it is possible now to process only the detached palm fruitlets via microwave heat (Hadi *et al*., 2021), but its practical implementation needs to be substantiated with an efficient mechanism for fruitlets separation from the bunches. Further processing of palm kernel cake from a crusher via solid-state fermentation could increase the crude protein content to serve as

a more nutritive mono-gastric feed material (Mohd Firdaus *et al.*, 2020). Besides, the non-compliant finding of odour emission limit of 12 000 OUm^{-3}, as proposed for assessment at source and *in-situ* palm oil mill sites, calls for an urgent counter proposal to fully comply and mitigate the sensory annoyance complaints by the public (Chung *et al.*, 2021).

The incorporation of POFA, in 10 wt.% as cement replacer, and POC, 50 wt. % as sand replacer has a potential to form a structured foamed concrete with the required strength (Abraham *et al.*, 2021). POC can also be made into nanoparticles to enhance the compressive strength of concrete (Hamada *et al.*, 2021). Its presence as sand replacer together with fly ash and furnace slag as a binder are promising in making cement and geopolymer mortars (Darvish *et al.*, 2021). The resultant cement exhibits up to 20% lighter weight with higher strengths. POFA is also a potential clay replacer (at optimum 10 wt.%) in brick manufacturing (Tjaronge and Caronge, 2021). These innovations help resolve POFA and POC disposal issues, conserving resources and maximising their utilisation, to yield more environmentally-friendly innovative construction materials. In addition, for the first time, POFA is also used as a cheap carbon precursor for the production of economical graphene nanosheets via a single-step chemical reaction. The highly porous graphene material is 70 times higher in its surface area (*i.e.*, from 22 $m^2\ g^{-1}$ to 1506.60 $m^2\ g^{-1}$) (Ayub *et al.*, 2021). On the other hand, 0.3 wt.% of EFB can serve as a stabiliser, for reinforced stone matrix asphalt concrete production (Yaro *et al.*, 2021). Active exploitation of these by-products can contribute to the sustainable production of construction materials.

Palm-based biochar as adsorbent continues as a recent interest topic. By converting oil palm frond through process-dependent parameters, the resultant biochar, in particularly activated biochar, has a high capability for SO_2 adsorption from flue gasses of power plants and factories (Iberahim *et al.*, 2022). The used biochar can be regenerated thermally. Palm kernel shell (PKS)-based activated carbon (0.85 mm in size) can remove 98% of phenolic pollutants (Sahu *et al.*, 2021). Oil palm trunk is another feedstock of interest to the scientific communities. Powdered oil palm trunk can bind with rubberwood veneer via a one-step liquefaction process at 180°C hot pressing for 5 min to form bio-adhesives, with or without citric acid as a chelating agent (Choowang and Luengchavanon, 2021). Bio-succinic acid continues to be researched via whole slurry saccharification at a mild oxalic acid concentration by Bukhari *et al.* (2021) for yield improvement. Inorganic salt-pretreated EFB gives a high titer and yield of succinic acid via simultaneous saccharification and fermentation (Anwar *et al.*, 2021).

In gearing toward addressing fossil fuel depletion and rising fuel cost, the current focus is to seek alternative biofuels with outstanding stability and combustion behaviour. The immediate 'low-hanging fruit' approach is through blending palm oil/PKO with methanol or ethanol in the presence of co-solvent such as tetrahydrofuran (THF) to yield a single-phase liquid termed micro-emulsion fuel (Jin *et al.*, 2021). By practically adjusting the proportion of each fuel ingredient, the resultant blended fuel would achieve optimal performance for the desired fuel properties. A combined blend of palm methyl esters (palm biodiesel) with cottonseed oil methyl esters and petroleum diesel fuel at a 20/80 ratio (*i.e.*, B20) can act as compatible diesel fuel for use without engine modification (Jamshaid *et al.*, 2022). Besides, it is possible to increase the combustion efficiency of palm biodiesel by just adding a single water droplet (Masharuddin *et al.*, 2021). The emulsified fuel experiences four stages of micro-explosion due to larger water particles and higher hydrophilic-lipophilic balance, thus, performing excellently with a reduced particulate matter (soot) and NOx emissions. It has also been proven that the mandated B7 (7 wt.% palm biodiesel in 93 wt.% petroleum diesel) and higher blends up to 20 wt.% can be comfortably used at Malaysia's highlands as their cold flow properties are lower than the lowest temperature recorded for the past 10 years in these places (Jalil *et al.*, 2021).

To improve process sustainability, industrial oil residues such as the ROPOME have increasingly been sought as a biodiesel feedstock. As the oil is similar to palm oil for conventional alkali-catalysed transesterification, its maximum oil recovery for biodiesel conversion must be accomplished. Four different types of solvents: n-hexane, methanol, ethanol, and toluene have been attempted (Zulqarnain *et al.*, 2021), yielding 90% of ROPOME at 1:1 (v/v) n-hexane-to-POME ratio, 500 rpm, pH 10 and 25 min mixing time. Its conversation into methyl esters reached 93%. Its combustion efficiency, *i.e.* 10 wt.% (B10) blend can be greatly enhanced via enrichment of hydroxy gas in low-displacement engines (Duarte-Forero, *et al.*, 2021). Similarly, sludge palm oil from the milling process has also been made into biodiesel via an enzymatic process, employing a genetically modified *Aspergillus oryzae* lipase (0.2 wt.%) and 5:1 methanol-to-oil molar ratio at 45°C (Loh *et al.*, 2021). The process has managed to produce crude biodiesel with 94 wt.% ester content, and the crude glycerol produced has higher purity compared to those from other established technologies. Ng *et al.* (2021) further applied this approach for rural electrification by supplying the enzymatically produced biodiesel to the surrounding households and mill operators. The approach shows high feasibility: 29% return on investment and <4 years payback period. Palm

oil soap stock is another biowaste which can firstly be acidified to yield 91 wt.% free fatty acids, then esterified using an immobilised lipase (alginate-polyvinyl alcohol) to produce biodiesel (Muanruksa *et al.*, 2021). The biocatalyst employed can be recycled up to 16 times.

Besides, the anaerobic digestion of POME for biogas production remains opportunistic as a form of renewable energy. Biogas capturing remains relevant in addressing greenhouse gas emissions associated with POME degradation. As of December 2020, a cumulative total of 130 biogas plants were on stream in Malaysia (Parveez *et al.*, 2021). This has increased to 135 plants in 2021 (unpublished data). One of the systems to treat POME is by integrating anaerobic (granular-sludge blanket) and aerobic (biofilm and activated sludge) processes, which can remove 99.7% of chemical oxygen demand (COD) (Show *et al.*, 2021). This approach generates revenue for combined heat and power as well as reduces greenhouse gas emissions. Moving forward, enhancement via co-fermentation has been made. Anaerobic co-digestion of POME with EFB for maximising biowaste value offers promising outcomes thus, far (Liew *et al.*, 2021). EFB must be pre-treated first prior to microbial degradation. Production of biogas through this approach at an optimal co-digestion ratio of EFB: POME, 0.6:1 is double that of mono-digestion under mesophilic conditions. Another integrated pilot-scale anaerobic–aerobic bioreactor loaded with an organic loading rate, *i.e.*, COD of 30.0 g L^{-1} day^{-1}, has successfully produced POME with biological oxygen demand (BOD) of <20 ppm consistently over 165 days (Yap *et al.* 2021a), besides enabling co-digestion with *Moringa oleifera* to enhance methane yield 1.5-fold (Yap *et al.*, 2021b). The protein-rich *M. oleifera* seed extract acts as a natural coagulant for enhanced microbial activity (Yap *et al.*, 2021b). The *M. oleifera* seed extract can also be made into magnetic nanoparticles via microwave and ultrasonic irradiation, which shows >75% removal of COD from POME (Noor *et al.*, 2021a). Another substrate, decanter cake, can double the methane yield when co-digested with POME (Lim *et al.*, 2021b). Nevertheless, Chan *et al.* (2021b) concluded that EFB exhibits a much-balanced C:N ratio for microbial rejuvenation, thus, offering better overall biogas enhancement when used as a co-substrate compared to decanter cake.

The other aspect which should be addressed when dealing with POME treatment is to remove the high organic strength BOD and COD before subjecting the final discharge into a waterway. Removal addresses pollutant load while nutrient recovery adds additional value to POME. Magnetic composite adsorbents show good performance in enhancing the POME treatment without having to go through polishing (Ratnasari *et al.*, 2022). A Fe-magnetised and activated carbon from palm kernel shell, having <250 μm particle size and 611.85 m^2 g^{-1} specific surface area, is able to polish and remove up to 99.7% and 85.0% of the initial colour and COD of raw POME, respectively (Tan *et al.*, 2021). Its reusability is great, with only <2.0% losses in removal efficiency after four reuse cycles. Furthermore, as POME is loaded with nutrients and beneficial microorganisms, research has also geared towards recovering and utilising it as a co-substrate for enhancing microbial degradation. Some of the examples exploited are as described in Ani *et al.* (2021) and Sayed *et al.* (2021), where POME has been shown to serve as a potential bio-stimulant in hydrocarbon degradation of contaminated soils, and for biodegradation of petroleum oil spills in shoreline, with an efficiency of 95.0% after 40 days. POME remains a relevant cultivation medium for microalgae growth targeting phytonutrients particularly, astaxanthin production, as well as pollutant bioremediation (Fernando *et al.*, 2021). Interestingly, the microalgae exploited, *Haematococcus pluvialis*, shows better adaptability in 7.5 wt.% POME concentration, compared to a lower, 5.0 wt.%, concentration for *Chlorella vulgaris* as a feedstock for biodiesel production three years ago (Idris *et al.*, 2018). Inorganic phosphorus can be recovered from POME while producing hydrogen via gasification in supercritical water (600°C, 25 MPa) (Mainil and Matsumura, 2021). Besides, a statistically optimised microbial electrolysis cell achieved a maximum production rate of 1.1747 m^3 hydrogen per 1 m^3 POME and can be performed daily (Kadier *et al.*, 2021).

The combustion behaviour of oil palm biomass determines the suitability of its conversion into solid biofuels making it an area worth investigating. A high potassium (K) content of the biomass usually causes slagging and fouling of power plants; thus, its removal is necessary before rendering the biomass for practical use to generate electricity. In doing so, Nasrin *et al.* (2021) have demonstrated that a combined sieving and water spraying method can produce low-ash (1.58 wt.%) pellets from EFB that originally contained 4.07 wt% ash, along with ~50.00% K removal. In another study, the content of K in EFB could be reduced via hydrothermal treatment prior to anaerobic digestion (biogas) and combustion in a boiler (electricity) (Saritpongteeraka *et al.*, 2021). Although >90.00% removal of K and higher methane production are achievable for a longer reaction time, only the former is able to recover ~70.00% residual oil. Judging from the energy and mass balances, the former shows a better advantage for incorporation into the existing milling practices.

Deoxygenation is paramount for producing advanced biofuel. An acid-base catalyst, such as acid-activated spent zeolite-based catalyst

recovered from an industrial cracking process (Istadi *et al.*, 2021), and zeolite beta (Nur Azreena *et al.*, 2021) employed during palm oil and oleic acid hydrocracking process, respectively, produced low-oxygenated hydrocarbon fuel (green diesel) with a composition similar to that of petroleum fuel. This type of high-quality biofuel can be directly used, with excellent corrosion resistance, stability and heating value. On the other hand, high-quality bio-hydrogenated diesel can be processed from palm oil with the help of Rh/HZSM-5 catalyst in a reduced hydrogen environment, for <1 min residence time (Kaewchada *et al.*, 2021). The high amount of hydrogen released during conventional hydro-treating of palm oil can be recovered (46%); the feasibility of hydrotreating can be improved (23% internal rate of return, 2.8 years payback period) (Phichitsurathaworn *et al.*, 2021).

Biojet fuel is worth researching due to fast-growing sustainable aviation fuel demand. Catalytic conversion using Ni/desilicated mesoporous zeolite-based catalyst yields biojet fuel from palm oil (Panarmasar *et al.*, 2021). The bioconversion and selectivity of jet-fuel products rely on the type of catalyst, reaction temperature and pressure. CPKO is another feedstock subjected to deoxygenation, employing Pt/Pd supported on activated carbon (Makcharoen *et al.*, 2021). A moderate yield of 58% of biojet fuel is attainable, of which 28% are linear alkane (C8-C16). Additional adjacent catalytic cracking and aromatisation process by HZSM-5 can lower its freezing point by 30°C.

Environmental Sustainability of Oil Palm Value Chain

In assessing environmental sustainability, life cycle assessment (LCA) serves as a promising tool, as seen with bourgeoning R&D activities in this area, be it for a technology, product or system. A pilot-scale up-flow anaerobic sludge blanket fixed-film reactor for biohydrogen production from POME was subjected to on LCA (Akhbari *et al.*, 2021). The resultant global warming impact by electricity usage can be further reduced to 54.9 kg CO_2 eq kg^{-1} H_2 if a cleaner electricity source can be identified and the burden in POME treatment avoided. In another study, Julio *et al.* (2021) showed that biodiesel and biojet fuel produced in an integrated palm oil biorefinery could avoid 24.65 kg CO_2 eq t^{-1} and 3281.36 CO_2 eq t^{-1}, respectively, compared to their fossil counterparts.

While it is beneficial to focus on LCA for large-scale palm oil processing, it is equally critical to conduct a gate-to-gate LCA of Nigerian mills with varying technological levels: large-, semi-mechanised, and smallholder-owned scales (Anyaoha and Zhang, 2021). Interesting findings include the existence of different hot spots in greenhouse gas emissions. While smaller-size and semi-mechanised mills emit 47% and 73% more CO_2 and N_2O, respectively than large-scale mills due to open burning of biomass residues and high diesel consumption, the latter emit 71% more methane due to inefficient EFB and POME management.

In a pioneering social-LCA study of CPO production, five palm oil companies located in Peninsular Malaysia show above average performance in fulfilling the eight subcategories of worker's indicators and basic requirements using the Subcategory Assessment Method, except for working hour (Haryati *et al.*, 2022). Long working hours are the social hot spots identified for further improvement, and the proposed mitigation is to train workers for the desired skills and also to incorporate advanced technology such as mechanisation. To better demonstrate environmental sustainability, all biomass residues generated from palm oil mills should be treated as by-products, which can be recycled and reutilised either as a fuel, mulching materials or fertilisers (Subramaniam *et al.*, 2021).

As palm oil production has been scrutinised for causing a high risk of indirect land-use change, reforming land policy is essential to enable oil palm planters to share their productive lands with those landless/displaced farmers (Azhar *et al.*, 2021). Such strategy has been shown via Monte Carlo simulations to mitigate land clearance and encourage intercropping and livestock integration.

Food Safety and Quality Research and Measures

Food safety and quality related issues remain important factors affecting consumers' food buying decisions worldwide. Increasing awareness and accessibility to a safe and healthy food supply are essential for the palm oil industry to deal with expectations from demanding consumers since palm oil is the most heavily traded edible oil. The situation has become direr for the industry and more highly relevant due to an ever-increasing demand to meet food safety regulations and quality standards. The most resilient issue at hand is how to address the inadvertent occurrence of 3-monochloropropane-1,2-diol esters (3-MCPDEs) and glycidyl esters (GEs) contaminants during the refining process of palm oil. Serious efforts are in place by the industry to eliminate these contaminants, in particular with the adoption of the Code of Practice (COP) in refined oils and food products made with refined oils, as adopted in CXC 79-2019 by the CODEX Alimentarius Commission (FAO, 2019).

Several mitigation strategies for 3-MCPDEs and GEs in palm oil have been reported by Tivanello *et al.* (2021). Reduction of these compounds in food products by reducing agents (0.05%, w/w) during

toasting (Belkova *et al*., 2021) and food preparation processes (Huang *et al*., 2021) are possible. Abd Razak *et al*. (2021) found that palm olein performed better compared to other oils such as soybean and canola oils, with a significant reduction of 3-MCPDE and GE content, while acrylamide concentration was dependent on the oil type and lipid oxidation profile. In a comprehensive review by Ahmad Tarmizi and Kuntom (2021), an in-depth deliberation on various parameters in relation to frying conditions and properties affecting the occurrence of these contaminants in vegetable oils was discussed. The varying observations further warrant explorations on the effects of different types of frying procedures, including food load and frying cycles.

The importance of detecting these compounds in vegetable oils and foods led to an increased number of studies on improvements in detection methods, including rapid detection methods (Martin *et al*., 2021), and simultaneous determination of esterified 2-/3-MCPD and glycidol in foods by GC-MS/MS (Zheng *et al*., 2021). A new approach using molecularly imprinted label-free sensor platform for impedimetric detection of 3-MCPD was also proposed (Yaman *et al*., 2021). Shaari *et al*. (2021) on the other hand reported a detection and extraction method for bound 3- and 2-MCPD and glycidol using accelerated solvent extraction and GC/MS. Besides, Sulaiman *et al*. (2021b) developed and validated a method for measuring residual 2,4-dichlorophenoxyacetic acid (2,4-D) herbicide in CPO using LC/MS/MS. The developed method is simple, economic, reliable and serves as an important tool for the regulatory monitoring of 2,4-D in palm oil. The successful combination of any available methods could be applied to the determination of the above-named contaminants in selected food products.

Studies in relation to other contaminants, apart from 3-MCPDE and GE, are also of importance in the efforts to address food safety issues of palm oil. Ahmad *et al*. (2021c) investigated the effect of different vegetable oils and frying cycles on the formation of acrylamide during intermittent frying of beef nuggets. Four different vegetable oil types (palm olein, red palm olein, sunflower oil and soybean oil) were compared in an 80-cycle frying experiment. It was found that the oil type but not the frying cycles affected acrylamide concentration in beef nuggets. The fate of acrylamide in the presence of Vitamin A and E homologues was also investigated by Kuek *et al*. (2021). An equimolar Asparagine-Glucose model system was used to evaluate the influence of Vitamin A and E homologues on acrylamide formation. The study reported that different Vitamin A and E concentrations could determine their functionality either as anti-oxidants or pro-oxidants. Further studies to look at the combined effects of the homologues on acrylamide formation are warranted.

The quality of commercial palm-based cooking oil in two types of packaging materials, the plastic pouch/packet and polyethylene terephthalate (PET) bottle, was compared in a study by Hassim *et al*. (2021a) to address the concern and misconception regarding the subsidised plastic pouch/packet oil which is cheaper than the oil sold in PET bottles. The study concluded that all parameters tested in the oils from both packaging were within the cooking oil specifications or guidelines set by both the Malaysian Standard (MS) 682:2004, MS 816:2007 and Palm Oil Refiners' Association (PORAM). This helps to clear the concern and misconception regarding the poorer quality of subsidised oil *vis-à-vis* those sold in PET bottles.

Food and Feed Research

As the most versatile oil for food manufacturing, there have been continuous efforts undertaken to valourise palm oil, PKO and their fractions in edible food uses. Palm oil, being semi-solid in nature, confers the desired characteristics and remains the exceptional choice for the cooking and baking industry, in providing the right fats such as margarines, shortenings and vanaspati. PKO on the other hand is the preferred fat for confectionary use due to its high lauric acid content and sharp melting properties. Likewise, the many fractions of palm oil and PKO have been continuously dissected and explored for innovations in the food as well as feed industry.

Hassim *et al*. (2021b) reported the use of blended palm fractions such as palm mid fraction (PMF) iodine value (IV) 45, PKO and palm stearin (POs) IV 33 and IV 14 as cocoa butter alternatives for chocolate bar production. The authors after testing eight different optimised blend ratios between solid fat content and varying temperatures reported a successful identification of the most suitable alternative fat for cocoa butter for the chocolate bars which would benefit the confectionery industry. In another study, Chaijan and Panpipat (2021) used pre-neutralised crude palm oil (NCPO) as a natural colourant and bioactive ingredient in tilapia fish sausages. It was found that 50 g/100 g substitution of NCPO for commercial refined palm oil could serve as a substitute for healthier tilapia sausage production.

A study by Kanagaratnam *et al*. (2021) documented the quality characteristics of retail refrigerated and non-refrigerated margarines and fat spreads, which are commonly used by Malaysians. Such information will be useful to consumers, especially when vast variations were observed in the characteristics and functionalities of these different fats in relation to their storage

and handling temperatures. It was noted that the refrigerated margarine and fat spreads were predominantly imported while all non-refrigerated ones were locally produced. Palm oil-based fats are the right choice as a replacer for partially hydrogenated fats in margarine/fat spreads as they can deliver the desired functionality with a reduced *trans* fatty acid content. The authors, in addition, reported a lower slip melting point, denoting a lower saturated fat content in refrigerated fats, compared to the non-refrigerated counterpart.

Innovations in food uses of palm oil will continue to be explored due to its unique properties and versatility. At this juncture, it is crucial to deliberate on the use of palm oil and its products in the feed industry. Saminathan *et al*. (2021) discussed the potential utilisation of treated oil palm frond (OPF) to improve feedstuff for ruminants. While the OPF could be an important source of feedstuff for ruminants, it confers several limitations, such as low metabolisable energy and high lignocellulosic content. Various pre-treatment approaches and the respective challenges warrant further enhancement of OPF nutritive values, in terms of biological and economic aspects.

For improvement in ruminant feed formulation, Ibrahim *et al*. (2021) reviewed the effects of vegetable oil supplementation on rumen fermentation and microbial population in ruminants. The authors discussed in detail the physiology of nutrient digestion in ruminants in relation to improvements in rumen fermentation and the distribution of microbes following supplementation with vegetable oils. Interestingly, it was found that the oil type caused no different effects towards fermentation in the rumen. However, oils which contain higher unsaturated fatty acids tend to show increased inhibition of the bacterial population in the rumen.

Palm Oil Nutrition Research

Palm oil nutritional research remains one of the key focus areas, especially because palm oil is mainly used in the food industry. Consumers are becoming more health conscious and are demanding the availability of reliable scientific evidence when selecting their choice of healthy foods. As such, extensive studies have been published to further strengthen the nutritional properties of palm oil and its loaded phytonutrients.

In 2021, several publications reviewed the effects of oil consumption on various disease conditions. One such study was on the role of dietary fats in inducing obesity-related postmenopausal breast cancer and the insights from mouse models (Tan and Teng, 2021). The authors highlighted the importance of choosing a suitable and reliable animal model, particularly postmenopausal breast cancer mouse models based on the biochemical mechanism related to the said condition. The effects confer on other parameters such as adipocytes, inflammatory mediators and related signalling molecules which are involved in the process. In addition, it was stressed that the types of dietary fats also play an important role in the development of postmenopausal breast cancer. Among the studies reviewed, there was no indication of a specific model which is best suited for conclusive effects on postmenopausal breast cancer. However, the authors suggest the establishment of new xenograft models which may serve as a more reliable model for future studies.

Moreover, Yap *et al*. (2021d) discussed the association of various dietary fats on the profile of gut microbiota and the possible effects of the palm oil diet. The review described the variation observed in the gut microbial population with the consumption of different dietary fats. As an example, there was less bacterial diversity when the saturated fatty acids rich diet was consumed. The bacterial diversity only increased with the monounsaturated fatty acids diet, and with the polyunsaturated fatty acids diet, a wider variation in bacterial diversity was observed. More studies are required to establish the microbial distribution in relation to palm oil consumption as the oil has a unique and balanced composition.

Several studies investigating the various fractions of palm oil were focused upon in 2021. Nagapan *et al*. (2021) explored the role of interesterified (IE) fats on lipid sub-fractions and hepatic gene expression involved in lipoprotein regulation, using F1B male Golden Syrian hamster (*Mesocricetus auratus*) model. Over a period of 12 weeks, the animals were fed high-fat diets *ad libitum*, containing 0.1% dietary cholesterol and 30.0% energy from dietary fat. The fats were either native or interesterified, namely, palm olein (PO), chemically interesterified palm olein (CIEPO), sal fat (from *S. robusta*) blend (SFB), and chemically interesterified sal fat blend (CIESFB). Plasma lipid profiles including low density lipoprotein (LDL) and high-density lipoprotein (HDL) sub-fractions, and hepatic gene expression levels were analysed. PO- and CIEPO-fed hamsters had 38.0% and 27.0% higher plasma HDL levels compared to SFB and CIESFB, respectively.

In addition, the authors also reported a greater proportion of the larger HDL particles in the PO diet fed animals, compared to those which received SFB and CIESFB diets. Whereas, animals fed with SFB and CIESFB had a greater proportion of larger LDL particles, compared to both palmitic counterparts. All diets have upregulated genes involved in liver fat accumulation. Palmitic-rich diets presented significant upregulation for the APO A1 gene ($p<0.05$). LDL metabolism related

genes such as LDLR, PCSK9, APO B, CYP7A1, PCSK9 were downregulated in all diets. In conclusion, native and IE saturated high-fat diets have induced liver steatosis in hamsters. The effects on plasma level HDL cholesterol and large HDL sub-fractions, however, were only seen in palmitic rich fats. Instead, the LDLR mediated cholesterol clearance was downregulated, with suppression of LDLR gene with similar effects on plasma LDL in all diets.

Voon *et al*. (2021a) reported on two studies on a specific fraction of palm oil *i.e.*, PMF, which is rich in 1, 3-dipalmitoyl-2-oleoglycerol (POP) triacylglycerol (TAG), produced by re-fractioning palm olein or palm stearin. The first study examined the role of PMF, equivalent to cocoa butter, as an alternative for healthier fat. The findings from the study indicated similar effects from the fats tested on postprandial lipoprotein metabolism, glycemia and insulinemic response. The second study by Voon *et al*. (2021b) reported on the effects of PMF on adult satiety, an indicator for reduced hunger and subsequently reduced food intake. The results showed that the PMF diets and high oleic sunflower diet have increased glucose dependant insulinotropic polypeptide (GIP) that may induce satiety response in human adults.

Over the years, the scientific evidence around fats and oils has mounted on the detrimental effects of *trans* fatty acids, especially on the increased risk of coronary heart disease (CHD) and mortality from CHD among others. A global exercise was initiated by the World Health Organization to eliminate industrially produced *trans* fatty acids by partial hydrogenation. A majority of the countries has started implementing various strategies to either prohibit the use of partially hydrogenated oils and/or limiting the levels of *trans* fatty acids. Palm oil has been the preferred choice to mitigate *trans* fatty acid occurrence in various food applications, because it does not require hydrogenation which is the major cause of the formation of *trans* fatty acids (Parveez *et al*., 2020). Nevertheless, there is also very limited data on the levels of *trans* fatty acids in cooking oils in Malaysia. Hishamuddin *et al*. (2022) studied the distribution levels of *trans* fatty acids in refined palm-based oils and other commercial vegetable oils in Malaysia. The findings showed that the palm-based cooking oils in Malaysia were superior to other oils, in that the levels of *trans* fatty acids were low, thereby meeting the regulatory levels of *trans* fatty acids of 1 g/100 mL.

On the palm phytonutrient research front, several studies were published on the mechanisms of action by tocotrienols in inhibiting breast cancer cell growth both *in vitro* and *in vivo*. Loganathan *et al*. (2021) reported that tocotrienols have exhibited anti-proliferative effects on human breast cancer cells by promoting programmed cell death or apoptosis possibly through the inactivation and down-regulation of two significant markers: poly-(ADP)-ribose polymerase-1 (PARP-1) and cyclooxygenase-2 (COX-2). In a breast cancer mice model, gamma-tocotrienol supplementation suppresses the growth of breast cancer tumour, as well as metastasis (Subramaniam *et al*., 2021a). The regulation of the immune system in the breast cancer mice model describes the mechanism of action imposed by gamma-tocotrienol (Subramaniam *et al*., 2021b).

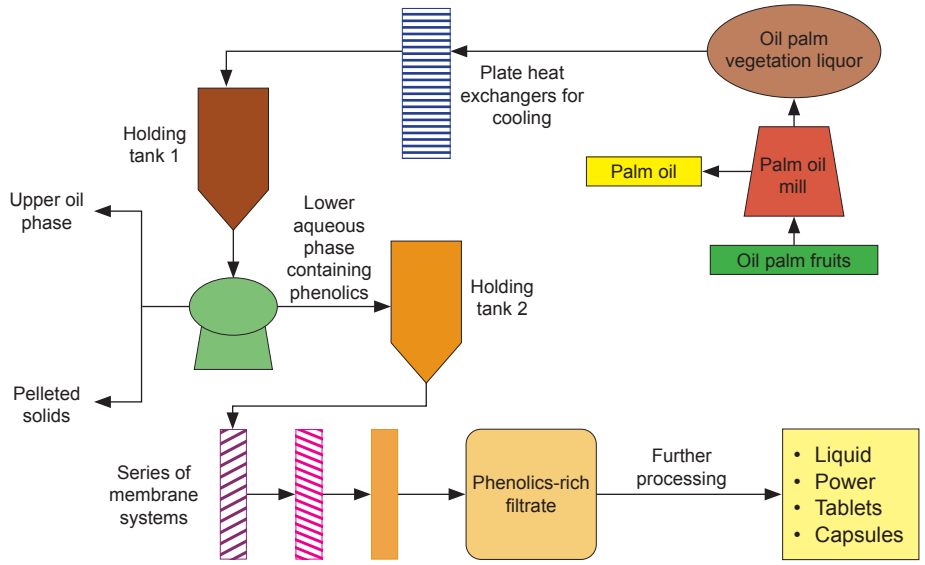
Research on water-soluble palm phytonutrients is another frontier in palm oil nutrition research. In 2021, Leow *et al*. (2021a) described the many facets of this unique compound, named water-soluble palm fruit extract (WSPFE), found in the aqueous extract of the palm fruit (*Figure 5*). The review described the complex compositions of the compound, and its biological properties as well as an in-depth discussion on its health and non-health applications. In another study, Leow *et al.* (2021b) investigated the possible neuroprotective effect of WSPFE as potential inhibitor of cholinesterase enzyme, which is crucial for the symptomatic treatment of Alzheimer's disease. The study found that WSPFE inhibited the cholinesterase and related enzymes, which warrants further exploration through *in vivo* models, as the way forward.

Oleochemical Innovation

The global growth of oleochemical market size is driven by the increasing consumption of renewable and sustainable bio-based chemicals in cosmetics, personal care, lubricants, polymers, pharmaceuticals, food, and other industries. The same 'green' market demand is also driving innovations in the development of products that utilise oleochemicals such as biolubricants, surfactants, bio-polyol, glycerol derivatives, agrochemicals and polymers.

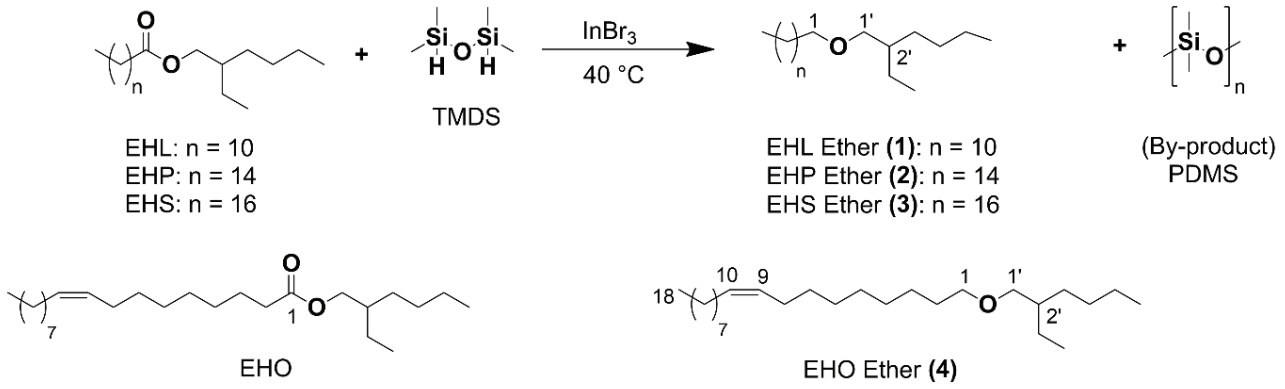
One of the focus areas of innovation is bio-lubricant, made from palm-based oleochemicals. In general, the use of bio-lubricants from renewable sources is gaining momentum due to rising concerns over the use of toxic and non-renewable mineral oil counterparts (Cecilia *et al*., 2020). Besides being environmentally-friendly, non-toxic and renewable, bio-lubricants have a higher flash point and very low volatile content, which make them safer to be used commercially.

However, several drawbacks such as poor oxidation stability and inferior cold flow properties, limit their usage in mainstream lubricant applications (Salih and Salimon, 2021). These drawbacks can be overcome through structural modification, as studied by Hoong *et al*. (2022), by producing estolide esters from palm-based oleic acid and lauric acid. Specifically, oleic acid is converted



Source: Leow *et al.* (2021a).

Figure 5. Water Soluble Palm Fruit Extract (WSPFE) production process.



Source: Tang *et al.* (2021).

Figure 6. Reduction of 2-ethylhexyl fatty esters to 2-ethylhexyl fatty ethers.

to estolide with hydroxyl groups through a reaction with hydrogen peroxide. Subsequently, hydroxyl groups of estolide are transformed into ester groups by end-capping them with organic acids of different chain lengths and structures. It was concluded that estolide esters, end-capped with lauric acid and 2-ethylhexanoic acid exhibited excellent cold flow properties with pour point as low as -36°C, and good oxidation stability with oxidation onset temperatures ranging from 193°C-200°C.

Another study by Tang *et al.* (2021) revealed that a series of 2-ethylhexyl alkyl ethers can be prepared from palm-based fatty esters through a reduction step catalysed by indium bromide in mild reaction conditions as shown in *Figure 6*. The synthesised bio-lubricants exhibited low kinematic viscosity of 4-9 cSt at 40°C, suggesting they are suitable for use in lubrication applications for fuel economy enhancement and energy efficiency. Additionally, the prepared bio-lubricants also showed good cold flow properties, with the lowest pour point of -27°C, they are most desirable for cold climates. The developed bio-lubricants can be employed across the supply chain of the palm oil industry, especially in palm oil mills, to substitute conventionally used mineral oil-based lubricants. These bio-lubricants not only incur low risk in the event of incidental contact with oil palm fruits or palm oil during harvesting and processing but are also more environmentally friendly, in case of any accidental release into the environment. Additionally, they could potentially be more cost-effective in the long run, compared to imported bio-lubricants.

Another focus area of innovation is the specialty chemicals, which can be derived from the common oleochemical glycerol. It contains three hydroxyl

groups for chemical modification to generate many high value-added bio-chemicals such as glycerol acetals that can be used as a fuel additive, surfactant and solvent. Instead of employing previously adopted homogeneous catalysts, Armylisas *et al.* (2021) employed bio-based aldehydes and the heterogenous acid catalyst, Amberlyst-46, to prepare glycerol acetals under solvent-free conditions, to simplify subsequent product purification steps which finally eliminate the use of solvent. The product yield and catalytic selectivity were influenced by the molecular structure and carbon chain length of the bio-based aldehyde. High yield was achieved using aldehydes with short carbon chain length such as acetaldehyde, while aromatic and longer chain length aldehydes gave low yield. Furthermore, the heterogeneous catalyst was shown to exhibit good reactivity and stability even after 10 reaction cycles. The prepared glycerol acetals have good potential to be used as a bio-based solvents to replace hazardous and non-renewable petroleum-based solvents due to their low toxicity and environmentally benign properties.

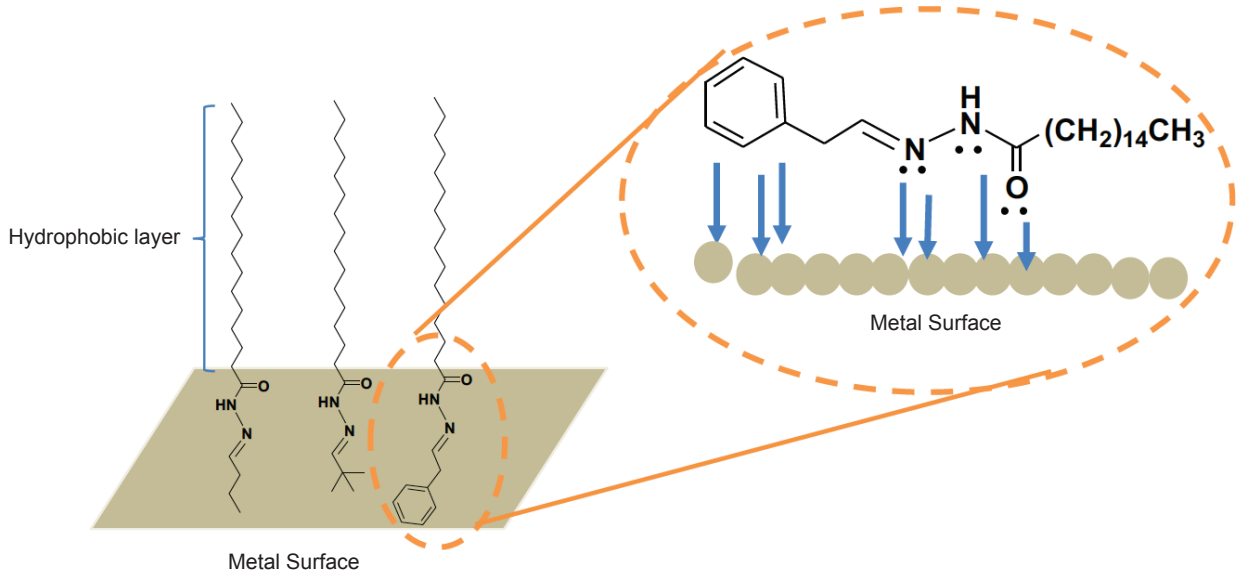
In another development, Mariam *et al.* (2021) reported on the synthesis of solketal levulinate ester, which can potentially be used as a potential additive to improve the properties of biodiesel such as cold flow, combustion profile and cetane number. In this study, the synthesised additive is wholly bio-based, using starting materials *i.e.*, solketal derived from glycerol, and methyl levulinate derived from biomass. The solketal levulinate ester with 95% purity at 75% yield will be applicable for biodiesel fuel upgrading.

The demand for renewable and sustainable chemicals is also observed in the field of polymeric materials such as bio-based polyol and polyurethane. These polymers have experienced significant growth in market share due to increasing demand for 'green' construction materials and renewable polymers in the automotive industry (Grand View Research, 2021). In response to such market demand, Noor *et al*. (2021b) synthesised palm-based polyols using both homogeneous and heterogeneous catalysts. The study revealed that polyol with a higher degree of oligomerisation, higher viscosity and higher hydroxyl functionality was attainable with $BF_3.EtO_2$ as the homogeneous catalyst, and the opposite properties were observed using K10-montmorillonite as the heterogeneous catalyst. This study gears towards the synthesis of palm-based polyol with specific properties for targeted application. In palm-based polyol's purification, the presence of impurity is crucial, *e.g.* sodium (Na) and K, as they might affect the reactivity of polyol with isocyanate for the preparation of polyurethanes. Therefore, the Na and K content in polyol are both limited to 10 mg kg^{-1}. In this regard, Ramli *et al*. (2021) developed a method to analyse Na and K content in palm-based polyols via graphite furnace atomic absorption spectroscopy. The developed method has been validated for routine analysis of Na and K contents in palm-based polyols.

Tailor-made palm-based thermoplastic polyurethane is another area worth innovating for a wide range of applications including coating, adhesive, sealant and elastomer. Ismail *et al*. (2021) reported that the co-monomeric polyester polyol-based thermoplastic polyurethane produced from palm-based azelaic acid in combination with succinic and adipic acids showed lower mechanical hysteresis (tensile) and tensile strength than those from conventional monomeric polyester polyol. The lower hysteresis of the prepared polymer suggested lower heat build-up, which is one of the desired properties for use in dynamic applications such as rollers and wheels.

R&D innovations for oleochemical derivatives have been extended into the field of thermal energy storage. Poopalam *et al*. (2021) studied the synthesis of 12 symmetrical fatty diamides from saturated fatty acids and aliphatic linear diamines. The study suggested that hydrogen bonding among fatty diamides is the key factor that affects the phase change properties of fatty diamides, and hydrogen bonding strength is mainly influenced by the chain length of diamine and fatty acid, which determine the molecular structure of fatty diamide and their crystallisation structures. The prepared fatty diamides were evaluated as phase change material (PCM), which can absorb and release thermal energy during phase transitions, which enables it to function as thermal energy storage material. Analysis results showed that the prepared fatty diamides broaden the range of operational temperature, with a melting point which is 80°C higher than current vegetable oil-based PCMs. Furthermore, the fatty diamides also exhibited better latent heat (220 J g^{-1}), thermal stability (380°C), and heat conductivity in comparison with current bio-based organic PCMs. These results suggested that the fatty diamides can be used as thermal energy storage materials for conservation of solar energy, re-cycling waste heat from industrial processes, improvement of thermal efficiency of buildings, and electric vehicles.

Mohd *et al*. (2021a) studied the physicochemical and electrical insulating properties of palm-based products such as RBD palm olein (RBDPOo), fatty acids, fatty esters and glycerol as the electrical insulating medium in oil-filled transformer for electrical energy transmission. RBDPOo was found to exhibit a high flash point of 320°C and an appropriate kinematic viscosity of 40 cSt at 40°C, which conformed to the required ASTM D6871 standard specification. The study also revealed that the dielectric breakdown strength of RBDPOo can be improved from 31.1 kV to 76.8 kV, by reducing the moisture content of RBDPOo from



Source: Mohd *et al.* (2021b).

Figure 7. The proposed adsorption sites of hydrophobic-tailed palm-based imines inhibitors on carbon steel surface in an acidic solution through π-electrons and lone pair electrons of heteroatoms.

400 ppm to 100 ppm. The blending of RBDPOo with fatty esters or fatty acids not only improved the kinematic viscosity of the blend but also enabled the blend to achieve a better flash and fire point than conventional mineral oil. This study suggested that palm oil products namely RBDPOo, fatty esters and fatty acids have good potential to be used as insulating oils for oil-filled transformers with better environmental footprint and fire-proof features than mineral oil.

Renewable, sustainable and environmentally friendly bio-based corrosion inhibitor is another focus area of oleochemical innovation due to rising demand for low toxicity, 'green' and cost-effective corrosion inhibitors by several industries. As a response to the demand, Mohd *et al.* (2021b) conducted a study to synthesise imine compounds from palm-based fatty acids and evaluated the synthesised compounds as corrosion inhibitors for carbon steel in hydrochloric acid solution. The imine compounds were prepared from a reaction between palmitic acid, hydrazine and various aldehydes with linear alkyl, branched alkyl and phenyl moiety, respectively.

Analysis of results from the study showed that palm-based imines prepared from aldehyde with linear alkyl and phenyl groups reduced the extent of corrosion damage on the surface of carbon steel exposed to the hydrochloric acid solution. The proposed adsorption sites of hydrophobic-tailed palm-based imines inhibitors on carbon steel surface in an acidic solution are illustrated in *Figure 7*. The hydrophobic layer originating from fatty acid protects the metal surface from being attacked by corrosive species. This study clearly indicated that palm-based imines have a high potential to be used as 'green' corrosion inhibitors for the protection of carbon steel.

Cosmetics and personal care products have been one of the main users of palm-based oleochemicals (Salimon *et al.*, 2012). Specifically, transparent soap is a beauty product sold in the upper-middle class market segment due to its luxurious appearance. Generally, plant extracts such as phenolics are included in the formulation of transparent soap to impart antioxidant properties that can prolong its shelf life. A study was carried out by Ahmad *et al.* (2021b), which incorporated oil palm leaf extracts containing phenolics into the palm-based transparent soap. The study showed that the addition of oil palm leaves extracts into transparent soap did not affect the foaming power and foam stability of the soap and improved the anti-oxidant properties of the soap, better than the commercial green tea extract transparent soap. Additionally, the prepared transparent soap with oil palm leaves extract exhibited natural yellow colour and showed stable hardness upon storage for 90 days. This study has shown that innovations could bring new zests in established technologies such as soap products and generate prospects for value creation.

Innovation in the oleochemical segment also extends into the engineering aspect of the oleochemical manufacturing technologies. Chan *et al.* (2021a) studied the synthesis of oleochemical derivative using microwave irradiation technology. A strategy to design a reactor and scaling-up the

synthesis of glycerol carbonate via microwave heating were assessed based on two parameters namely absorbed microwave energy density and absorbed microwave power density. Optimisation of both parameters enabled the determination of optimum microwave power requirement and heating duration for the synthesis of glycerol carbonate in a constant-power microwave heating mode. Findings from this study will enable further development of microwave heating technology such as continuous-flow microwave heating technology to produce oleochemical derivatives.

New analytical method development is also an important innovation to safeguard the quality of oleochemicals. Tay and Wai (2021) developed an analytical method to detect and quantify chlorpyrifos in food-grade palm-based fatty acids. Chlorpyrifos is a pesticide approved by the Malaysian Pesticide Board for use on oil palm trees to control related pests. There are worries that chlorpyrifos residues could be found in fatty acids after specific processing steps. Therefore, an analytical method was established to specifically detect and quantify chlorpyrifos in palm-based fatty acids by using a simple direct injection method, coupled with gas chromatography-mass spectrometry (GC-MS). The limits of detection and quantification of chlorpyrifos for this developed method were 0.5 μg g^{-1} and 5 μg g^{-1}, respectively. It is noteworthy that the level of chlorpyrifos found in commercial fatty acids was below 0.5 μg g^{-1}, the legally permitted level of pesticides in food commodities and animal feed in Malaysia. This result suggested that any chlorpyrifos residue found in commercial fatty acids will not be harmful to humans.

CONCLUSION

In general, the economic performance of the Malaysian palm oil industry in 2021 was very impressive, with many oil palm plantation companies reported healthy profits from their business operation. This was due to lower CPO production brought about by shortage of labour due to the COVID-19 pandemic, which restricted the supply of palm oil which in turn, favourably drove CPO prices to an all-time high. Despite the stellar economic achievement, continuous R&D efforts are being conducted in all sectors of the palm oil industry, to persistently enhance the well-being of the industry. In the upstream sector, precision agriculture technology, farm mechanisation, advanced breeding and genomics research as well as an effective pest and diseases controls contributed to the overall improvement of CPO yield while simultaneously enhancing the sustainability of oil palm plantation by minimising environmental impact. As for the midstream sector, R&D efforts were focused on improving the productivity of palm oil mill and mitigating the environmental impact of milling operations, especially relating to POME. Furthermore, R&D emphasis on the utilisation of palm-based biomass is a vital approach to unlock the potential value from by-products of the industry while moving towards a circular economy. The downstream sector of the industry also plays an important role in supplying the world with food and non-food products, that are not only safe but also sustainable. Therefore, R&D efforts have been intensified to mitigate any food safety issues related to palm oil in addition to efforts that were made to enhance the nutritious value of palm oil in food products. Moreover, R&D activities in producing sustainable and value-added non-food products from palm oil such as bio-fuel, bio-polyol, bio-lubricants and personal care products were strengthened to offer global consumers greener products as well as to increase the consumption of palm oil. Nonetheless, continuous efforts are needed to strengthen the palm oil industry, in its journey towards circular economy and sustainability.

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REFERENCES

Abd Razak, R A; Ahmad Tarmizi, A H; Kuntom, A; Sanny, M and Ismail, I S (2021). Intermittent frying effect on French fries in palm olein, sunflower, soybean and canola oils on quality indices, 3-monochloropropane-1, 2-diol esters (3-MCPDE), glycidyl esters (GE) and acrylamide contents. *Food Control*, *124:* 107887.

Abraham, H B; Alengaram, U J; Alnahhal, A M; Haddadian, A; Karthick, S and Deboucha, W (2021). Performance evaluation of cellular lightweight concrete using palm oil industrial waste as cement and fine aggregate replacement materials. *Mater. Today: Proc.*, 52: 902-910. DOI: 10.1016/j.matpr.2021.10.301.

Adon, B; Konan, J N; Cochard, B; Flori, A; Diabaté, S; Bakoumé, C and Sokouri, D P (2021). Agronomical performances of Angolan natural oil palm accessions and interests for oil palm selection in Côte d'Ivoire. *J. Agric. Sci.*, *13 (11)*: 64-73.

Ahmad, M N; Shariff, A R M; Aris, I; Halin, I A and Moslim, R (2021a). Identification and determination of the spectral reflectance properties of live and

dead bagworms, *Metisa plana* Walker (Lepidoptera: Psychidae) using VIS/NIR spectroscopy. *J. Oil Palm Res., 33(3):* 425-435.

Ahmad, N; Hasan, Z A A and Bilal, S H (2021b). Stability and performance of palm-based transparent soap with oil palm leaves extract. *J. Oil Palm Res., 33(4)*: 724-731. DOI: 10.21894/jopr.2021.0024.

Ahmad, S N S; Ahmad Tarmizi, A H; Abd Razak, R A; Jinap, S; Norliza, S; Sulaiman, R and Sanny, M (2021c). Selection of vegetable oils and frying cycles influencing acrylamide formation in the intermittently fried beef nuggets. *Foods, 10 (2)*: 257.

Ahmad Tarmizi, A H and Kuntom, A (2021). The occurrence of 3-monochloropropane-1, 2-diol esters and glycidyl esters in vegetable oils during frying. *Crit. Revi. Food Sci. Nutr., 62(12)*: 1-17. DOI: 10.1080/10408398.2020.1865264.

Ainul, S S; Ali, Z. A. N; Kamalrudin, M S; Amran A; and Rahmahwati, R (2021a). Factors affecting fresh fruit bunch yields of independent smallholders in Sabah. *Oil Palm Industry Economic J., 21(2)* : 22-34. DOI: 10.21894/opiej.2021.06.

Ainul, S S; Zaki, A; Abdul Rahman, A R; Nazirah, C J; Humaira, M T; Rahmahwati, R; Amran A; and Sohimi, M R (2021b). Factors influencing the performance of sustainable oil palm grower's cooperative in Sabah. *International Journal of Modern Trends in Social Sciences*, *4(16)*: 25-34. DOI: 10.35631/IJMTSS.416003.

Akhbari, A; Onn, C C and Ibrahim, S (2021). Analysis of biohydrogen production from palm oil mill effluent using a pilot-scale up-flow anaerobic sludge blanket fixed-film reactor in life cycle perspective. *Int. J. Hydrog. Energy, 46(68)*: 34059-34072.

Amit, B; Tuen, A A and Kho, L K (2021). Bird species richness, abundance, and their feeding guild across oil palms development through mist-netting method in Betong, Sarawak. *J. Oil Palm Res., 33 (4):* 617-628.

Ani, K A: Agu, C M and Menkiti, M C (2021). Preliminary investigation and neural network modeling of palm oil mill effluent as a potential bio-stimulating organic co-substrate in hydrocarbon degradation. *Environmental Challenges*, *5*: 100216.

Anwar, N A K K; Hassan, N; Idris, A; Ramli, S and Malek, R A (2021). Large scale production of succinic acid by fermentation of sequential inorganic salt pretreated oil palm empty fruit bunch. *Biomass Bioenergy, 155(3)*: 106307.

Anyaoha, K E and Zhang, L (2021). Renewable energy for environmental protection: Life cycle inventory of Nigeria's palm oil production. *Resour. Conserv. Recycl.*, *174*: 105797.

Apriyanto, A and Tambunan, V B (2021). Draft genome sequence, annotation, and SSR mining data of *Elaeidobius kamerunicus* Faust., an essential oil palm pollinating weevil. *Data Br.*, *34(7)*: 106745.

Armylisas, A H N; Yeong, S K; Maznee, T I T N and Hoong, SS (2021). Effect of bio-based aldehyde structure on the conversion and selectivity towards glycerol acetal over Amberlyst-46 by using a solvent-free approach. *ChemistrySelect*, *6(7)*: 256-263. DOI: 10.1002/slct.202004215.

Ayob, Z and Kusai N A (2021). A comparative study of bacterial communities determined by culture-dependent and-independent approaches in oil palm planted on tropical peatland. *J. Oil Palm Res., 33(4)*: 588-606.

Ayompe, L M; Schaafsma, M and Egoh, B N (2021) Towards sustainable palm oil production: the positive and negative impacts on ecosystem services and human wellbeing. *J. Clean. Prod.*, *278(3)*: 123914.

Ayub, M; Othman, M H D; Yusop, M Z M; Khan, I U and Zakria, H S (2021). Facile and economical, single-step single-chemical method for conversion of palm oil fuel ash waste into graphene nanosheets. *Appl. Mater. Today*, *25(80)*: 101193.

Azhar, B; Nobilly, F; Lechner, A M; Tohiran, K A; Maxwell, T M R; Zulkifli, R; Kamel, M F and Oon, A (2021). Mitigating the risks of indirect land use change (ILUC) related deforestation from industrial palm oil expansion by sharing land access with displaced crop and cattle farmers. *Land Use Policy*, *107*: 105498.

Azimi, N; Ooi, S E and Ong-Abdullah, M (2021). Evaluation of mitochondrial DNA isolation methods for oil palm (*Elaeis guineensis*) leaf**.** *J. Oil Palm Res., 33(4)*: 607-616. DOI: 10.21894/jopr.2021.0009.

Babu, K B; Mathur, R K; Anitha, P; Ravichandran, G and Bhagya, H P. (2021). Phenomics, genomics of oil palm (*Elaeis guineensis* Jacq.): Way forward for making sustainable and high yielding quality oil palm. *Physiol. Mol. Biol. Plants*, *27(3)*: 587-604. DOI: 10.1007/s12298-021-00964-w.

Bahariah, B; Masani, M Y A; Rasid, O A and Parveez, G K A (2021). Multiplex CRISPR/Cas9-mediated genome editing of the FAD2 gene in rice: a model genome editing system for oil palm. *J. Genet. Eng.*

Biotechnol., *19(1)*: 86. DOI: 10.1186/s43141-021-00185-4.

Behera, S K; Suresh, K; Shukla, A K; Kamireddy, M; Mathur, R K and Majumdar K (2021). Soil and leaf potassium, calcium, and magnesium in oil palm (*Elaeis guineensis* Jacq.) plantations grown on three different soils of India: status, stoichiometry and relations. *Ind. Crops Prod.*, *168 (3)*: 113589.

Belkova, B; Chytilova, L; Kocourek, V; Slukova, M; Mastovska, K; Kyselka, J and Hajslova, J (2021). Influence of dough composition on the formation of processing contaminants in yeast-leavened wheat toasted bread. *Food Chem., 338(1)*: 127715.

Bukhari, N A; Loh, S K, Luthfi, A A I, Abdul, P M, Nasrin, A B, Harun, S and Jahim, JM (2021). Whole slurry saccharification of mild oxalic acid-pretreated oil palm trunk biomass improves succinic acid production. *Ind. Crops Prod.*, *171*: 113854.

Cecilia, J A; Plata, D B; Saboya, R M A; Luna, F M T; Cavalcante, C L and Rodriguez-Castellon, E (2020). An overview of the biolubricant production process: Challenges and future perspectives. *Processes*, *8(3)*: 257. DOI:10.3390/pr8030257.

Chaijan, M and Panpipat, W (2021). Pre-neutralized crude palm oil as natural colorant and bioactive ingredient in fish sausage prepared from tilapia (*Oreochromis niloticus*). *LWT*, *135*: 110289.

Chan, C H; Manap, N I A; Din, N S M N M; Hazmi, A S A; Kow, K W and Ho, Y K (2021a). Strategy to scale up microwave synthesis with insight into the thermal and non-thermal effects from energy-based perspective. *Chem. Eng. Process.*, *168*: 108594. DOI:10.1016/j.cep.2021.108594.

Chan, Y J; Lee, H W and Selvarajoo, A (2021b). Comparative study of the synergistic effect of decanter cake (DC) and empty fruit bunch (EFB) as the co-substrates in the anaerobic co-digestion (ACD) of palm oil mill effluent (POME). *Environmental Challenges*, *5*: 100257.

Cheyns, E and Rafflegeau, S (2005). Family agriculture and the sustainable development issue: Possible approaches from the African oil palm sector. The example of Ivory Coast and Cameroon. *OCL Oilseeds and Fats, Crops and Lipids*, 12(2): 111-120. DOI:10.1051/ocl.2005.0111.

Choowang, R and Luengchavanon, M (2021). Oil palm trunk powder blended with citric acid directly used as wood adhesive by one step liquefaction reaction. *Ind. Crops Prod.*, *170(2)*: 113809.

Chung, A Y K; Zaman, N Q; Manaf, F Y A; Halim, R M and Majid, R A (2021). Palm oil mills odour emission survey based on different pome treatment system. *Jurnal Kejuruteraan*, *33(1)*: 113-131.

Darvish, P; Alengaram, U J; Alnahhal, A M; Poh, Y S and Ibrahim, S (2021). Enunciation of size effect of sustainable palm oil clinker sand on the characteristics of cement and geopolymer mortars. *J. Build. Eng.*, *44(3165)*: 103335.

Divinechocolate (2022). https://www.divinechocolate.com, accessed on 17 May 2022.

Donfack, L S; Ro¨ll, A; Ells¨aßer, F; Ehbrecht, M; Irawan, B; Ho¨lscher, D; Knohl, A; Kreft, H; Siahaan, E J; Sundawati, L; Stiegler, C and Zemp, C D (2021). Microclimate and land surface temperature in a biodiversity enriched oil palm plantation. *For. Ecol. Manag.*, *497*: 119480.

Duarte-Forero, J; Mendoza-Casseres, D and Valencia-Ochoa, G (2021). Energy, Exergy, and emissions (3E) assessment of a low-displacement engine powered by biodiesel blends of palm oil mill effluent (POME) and hydroxy gas. *Ther. Sci. Eng. Prog.*, *26(8)*: 101126.

Fahrizal, M; Sarifuddin; and Sabrina, T (2021). Potential of *Syncephalastrum racemosum* and *Rhizopus arrhizus* isolated from oil palm trunks produce chitosans that inhibit the growth of *Ganoderma boninense. J. Oil Palm Res., 33(2)*: 381-386.

FAO (2019). CODEX Alimentarius, CXC 79-2019. Code of practice for the reduction of 3-monochloropropane-1,2- diol esters (3-MCPDEs) and glycidyl esters (GEs) in refined oils and food products made with refined oils. https://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXC%2B79-2019%252FCXC_079e.pdf, accessed on 19 March 2022.

Fernando, J S R; Premaratne, M; Dinalankara, D M S D; Perera, G L N J and Ariyadasa, T U (2021). Cultivation of microalgae in palm oil mill effluent (POME) for astaxanthin production and simultaneous phycoremediation. *J. Environ. Chem. Eng.*, *9(4)*: 105375.

Filho, W R L L; Rodrigues, F H S; Ferreira, I V L; Correa, L O; Cunha, R L and Pinheiro H A (2021). Physiological responses of young oil

palm (*Elaeis guineensis* Jacq.) plants to repetitive water deficit events. *Ind. Crops Prod.*, *172*: 114052.

Fitzherbert, E B; Struebig, M J; Morel, A; Danielsen, F; Brühl, C A; Donald, P F and Phalan, B. (2008). How will oil palm expansion affect biodiversity?. *Trends Ecol. Evol.*, *23(10)*: 538-545.

Gan, S T; Teo, C J; Manirasa, S; Wong, W C and Wong, C K (2021). Assessment of genetic diversity and population structure of oil palm (*Elaeis guineensis* Jacq.) field genebank: A step towards molecular-assisted germplasm conservation. *PLoS One*, 16(7): e0255418. DOI: 10.1371/journal.pone.0255418.

Grand View Research (2021). Bio-based polyurethane market size, share and trends analysis report by product (rigid foam, coatings, adhesives and sealants, flexible foam), by end use (packaging, building and construction), and segment forecasts, 2021–2028. https://www.grandviewresearch.com/industry-analysis/bio-based-polyurethane-industry, accessed on 27 December 2021.

Hadi, N A; Han, N M; Majid, R A and Mat, C R C (2021). The effect of microwave treatment and delayed harvesting on oil palm fruitlets (*Elaeis guineensis*) oil quality. *J. Oil Palm Res., 33(4)*: 678-688. DOI: 10.21894/jopr.2021.0015.

Hamada, H M; Alattar, A A; Yahaya, F M: Muthusamy, K and Tayeh, B A (2021). Mechanical properties of semi-lightweight concrete containing nano-palm oil clinker powder. *Phys. Chem. Earth (2022), Parts A/B/C*, *121*: 102977.

Harith, H H: Mohd, M F and Sowat, S N (2021). A preliminary investigation on upper limb exoskeleton assistance for simulated agricultural tasks. *Appl. Ergon.*, *95*: 103455.

Haryati, Z; Subramaniam, V; Noor, Z Z; Loh S K and Aziz, A A (2022). Social life cycle assessment of crude palm oil production in Malaysia. *Sustain. Prod. Consum.*, *29*: 90-99.

Hashim, Z; Muhamad, H; Chan, K W; Choo, Y M and Wahid, M B (2010). Life cycle assessment for oil palm fresh fruit bunch production from continued land use for oil palm planted on mineral soil (Part 2). *J. Oil Palm Res., 22*: 887-894.

Hassim, N A M; Ismail, N H; Kanagaratnam, S; Isa, W R A and Dian, N L H M (2021a). Quality of commercial palm-based cooking oil packed in plastic pouch and polyethylene terephthalate (PET) bottle. *J. Oil Palm Res., 33(3)*: 493-513.

Hassim, N A M; Kanagaratnam, S and Dian, N L H M (2021b). Blended palm fractions as confectionery fats: A preliminary study. *J. Oil Palm Res., 33(2)*: 360-380.

Hervas, A (2021). Mapping oil palm-related land use change in Guatemala, 2003-2019: Implications for food security. *Land Use Policy*, *109*: 105657.

Hilal, Y Y; Yahya, A; Ismail, W I W and Asha'ari, Z H (2021). Neural networks method in predicting oil palm FFB yields for the Peninsular States of Malaysia. *J. Oil Palm Res., 33(3)*: 400-412.

Hishamuddin, E; Abd Razak, R A; Yeoh, C B and Ahmad Tarmizi, A H (2022). Assessment of *trans* fatty acid levels in refined palm-based oils and commercial vegetable oils in the Malaysian market. *J. Oil Palm Res., 34(1)*: 129-138. DOI: 10.21894/jopr.2021.0029.

Hoong, S S; Arniza, M Z; Mariam, N M D N S; Armylisas, A H N, Tang, S W; Ismail, T N M T and Yeong, S K (2022). Synthesis and physicochemical properties of new estolide esters as potential biolubricant base oil. *J. Oil Palm Res., 34(1)*: 152-166. DOI: 10.21894/jopr.2021.0032.

Huang, Z; Xie, D; Cao, Z; Guo, Z; Chen, L; Jiang, L; Sui, X and Wang, Z (2021). The effects of chloride and the antioxidant capacity of fried foods on 3-chloro-1, 2-propanediol esters and glycidyl esters during long-term deep-frying. *LWT*, *145*: 111511.

Hussin, S H; Seman, I A: Rusli, M H: Mohamed, M S and Sapak, Z (2021). Herbicide effects on *Ganoderma boninense* infection in oil palm seedlings. *J. Oil Palm Res., 33(1)*: 56-63.

Iberahim, N; Sethupathi, S; Bashir, M J K; Kanthasamy, R and Ahmad, T (2022). Evaluation of oil palm fiber biochar and activated biochar for sulphur dioxide adsorption. *Sci. Total Environ.*, *805(2020)*: 150421.

Ibrahim, N A; Alimon, A R; Yaakub, H; Samsudin, A A; Candyrine, S C L; Wan Mohamed, W N; Md Noh, A; Fuat, M A and Saminathan, M (2021). Effects of vegetable oil supplementation on rumen fermentation and microbial population in ruminant: A review. *Trop. Anim. Health Prod.*, *53(4)*: 1-11.

Idris, N A; Loh S K; Lau, H L N: Yau, T C; Mustafa, E M; Vello, V and Moi, P S (2018). Palm oil mill effluent as algae cultivation medium for biodiesel production. *J. Oil Palm Res., 30(1)*: 141-149.

Ishak, N A; Tahir, N I; Mohd Sa'id, S N; Gopal, K; Othman, A and Ramli, U S (2021). Comparative analysis of statistical tools for oil palm phytochemical research. *Heliyon*, *7*: e06048.

Ismail, T N M T; Ibrahim, N A; Palam, K D P; Sendijarevic, A; Sendijarevic, I; Schiffman, C M; Hoong S S; Noor, M A M; Yeong, S K; Malek, E A; Zainuddin, E and Sendijarevic, V (2021). Improved dynamic properties of thermoplastic polyurethanes made from co-monomeric polyester polyol soft segments based on azelaic acid. *J. Appl. Polym. Sci.*, *138*: 50815. DOI: 10.1002/app.50815.

Istadi, I; Riyanto, T; Khofiyanida, E; Buchori, L; Anggoro, D D; Sumantri, I; Putro, B H S and Firnanda, A S (2021). Low-oxygenated biofuels production from palm oil through hydrocracking process using the enhanced spent RFCC catalysts. *Bioresource Technology Reports*, *14*: 100677

Ithnin, M; Vu, W T; Shin, M G; Suryawanshi, V; Sherbina, K; Zolkafli, S H; Norhalida, M S; Mohd Din A; Abdullah, N; Mustaffa, S; Marjuni, M; Rajanaidu, N; Kushairi, A; Marjoram, P; Nuzhdin, S V; Chang, P L and Singh, R (2021). Genomic diversity and genome-wide association analysis related to yield and fatty acid composition of wild American oil palm. *Plant Sci.*, *304*: 110731.

Jalil, N; Lau, H L N and Jalal, R I A (2021). The cold flow properties of palm biodiesel for diesel blends mandate in Malaysia's highlands. *J. Oil Palm Res., 34(1)*: 116-128. DOI: 10.21894/jopr.2021.0027.

Jamshaid, M; Masjuki, H H; Kalam, M A; Zulkifli N W M; Arslan, A and Qureshi, A A (2022). Experimental investigation of performance, emissions and tribological characteristics of B20 blend from cottonseed and palm oil biodiesels. *Energy*, *239*: 121894.

Jaroenkietkajorn, U and Gheewala, S H (2021). Understanding the impacts on land use through GHG-water-land-biodiversity nexus: The case of oil palm plantations in Thailand. *Sci. Total Environ.*, *800*: 149425.

Jin, C; Liu, X; Sun, T; Ampah, J D: Geng, Z; Ikram, M; Ji, J; Wang, G and Liu, H (2021). Preparation of ethanol and palm oil/palm kernel oil alternative biofuels based on property improvement and particle size analysis. *Fuel*, *305*: 121569.

Jin, L; Yarra, R; Zhou, L and Cao, H (2022). The auxin response factor (ARF) gene family in oil palm (*Elaeis guineensis* Jacq.): Genome-wide identification and their expression profiling under abiotic stresses. *Protoplasma*, *259(1)*: 47-60. DOI: 10.1007/s00709-021-01639-9.

Julio, A A V; Batlle, E A O; Trindade, A B; Nebra, S A; Reyes, A M M and Palacio, J C E (2021). Energy, exergy, exergoeconomic, and environmental assessment of different technologies in the production of bio-jet fuel by palm oil biorefineries. *Energy Convers. Manag.*, *243*: 114393.

Kadier, A; Wang, J; Chandrasekhar K; Abdeshahian, P; Amirul Islam, M; Ghanbari, F; Bajpai, M; Katoch, S S; Bhagawati, P B; Li, H; Kalil, M S; Hamid, A A; Hasan, H A and Ma, P C (2021). Performance optimization of microbial electrolysis cell (MEC) for palm oil mill effluent (POME) wastewater treatment and sustainable Bio-H2 production using response surface methodology (RSM). *Int. J. Hydrog. Energy.*, DOI: 10.1016/j.ijhydene.2021.09.259.

Kaewchada, A; Akkarawatkhoosith, N: Bunpim, D; Bangjang, T; Ngamcharussrivichai, C and Jaree, A (2021). Production of bio-hydrogenated diesel from palm oil using Rh/HZSM-5 in a continuous mini fixed-bed reactor. *Chem. Eng. Process.*, *168*: 108586.

Kamu, A; Phin, C K; Seman, I A; Gabda, D and Mun, H C (2021). Estimating the yield loss of oil palm due to *Ganoderma* basal stem rot disease by using Bayesian Model Averaging. *J. Oil Palm Res., 33(1)*: 46-55.

Kanagaratnam S; Teng, K T; Ismail, N H; Hassim, N A M; Isa, W R A and Dian, N L H M (2021). Characteristics of retail refrigerated and non-refrigerated margarines/fat spreads sold in Malaysia. *J. Oil Palm Res., 33(4)*: 689-702. DOI: 10.21894/jopr.2021.0006.

Khai, C T Z; Hung, P K J; Ming, S C; Lian, H K and Hong, L W (2021). Expression of cadherin gene in *Bacillus thuringiensis* Cry1A toxin resistant and susceptible strains of *Tirathaba mundella*. *J. Oil Palm Res., 33(2)*: 248-256.

Kok, S Y; Namasivayam, P; Ee, G C L and Ong-Abdullah, M (2021). Comparative proteomic analysis of oil palm (*Elaeis guineensis* Jacq.) during early fruit development. *J. Proteomics*, *232*: 104052. DOI: 10.1016/j.jprot.2020.104052.

Krishna, V V and Kubitza, C (2021). Impact of oil palm expansion on the provision of private and community goods in rural Indonesia. *Ecol. Econ.*, *179*: 106829.

Kuek, S L; Ahmad Tarmizi, A H; Abd Razak, R A; Jinap, S and Sanny, M (2021). Vitamin A and E homologues impacting the fate of acrylamide in Equimolar Asparagine-glucose Model system. *Antioxidants*, *10(7)*: 993.

Kusumawati, S A; Yahya, S; Hariyadi; Mulatsih, S; and Istina, I N (2021). The dynamic of carbon dioxide (CO_2) emission and land coverage on intercropping system on oil palm replanting area. *J. Oil Palm Res.*, *33(2)*: 267-277.

Leow, S S; Fairus, S and Sambanthamurthi, R (2021a). Water-soluble palm fruit extract: composition, biological properties, and molecular mechanisms for health and non-health applications. *Crit. Rev. Food Sci. Nutr.* DOI: 10.1080/10408398.2021.1939648.

Leow, S S; Fairus, S and Sambanthamurthi, R (2021b). Inhibition of cholinesterases by water-soluble palm fruit extract. *J. Oil Palm Res.*, *34(1)*: 139-151. DOI: 10.21894/jopr.2021.0028.

Liew, Z K; Chan, Y J; Ho, Z T; Yip, Y H; Teng, M C; Ameer Abbas, A I T; Chong, S; Show, P L and Chew, C L (2021). Biogas production enhancement by co-digestion of empty fruit bunch (EFB) with palm oil mill effluent (POME): Performance and kinetic evaluation. *Renew. Energ.*, *179*: 766-777.

Lim, C H; Cheah, Z H; Lee, X H; How, B S; Ng, W P Q; Ngan, S L; Lim, S and Lam, H L (2021a). Harvesting and evacuation route optimisation model for fresh fruit bunch in the oil palm plantation site. *J. Clean. Prod.*, *307*: 127238.

Lim, Y F; Chan, Y J; Hue, F S; Ng, S C and Hashma, H (2021b). Anaerobic co-digestion of palm oil mill effluent (POME) with decanter cake (DC): Effect of mixing ratio and kinetic study. *Bioresource Technology Reports*, *15*: 100736.

Loganathan, R; Selvaduray, K R; Nesaretnam, K and Radhakrishnan, A K (2021). Palm tocotrienols cause cleavage of poly-(adp)-ribose polymerase enzyme and down-regulation of cyclooxygenase-2 protein level in human breast cancer cells. *J. Oil Palm Res.*, *33(1)*: 119-128.

Loh, J M; Amelia; Gourich, W; Chew, C L; Song, C P and Chan, E-S (2021). Improved biodiesel production from sludge palm oil catalyzed by a low-cost liquid lipase under low-input process conditions. *Renew. Energ.*, *177*: 348-358.

Mahmud, M S and Chong, K P (2021). Formulation of biofertilizers from oil palm empty fruit bunches and plant growth-promoting microbes: A comprehensive and novel approach towards plant health. *J. King Saud Univ. – Science*, *33*: 101647.

Mainil, R I and Matsumura, Y (2021). Effect of heating rate on gasification and phosphorus recovery for palm oil mill effluent in supercritical water. *J. Supercrit. Fluids*, *173*: 105217.

Makcharoen, M; Kaewchada, A; Akkarawatkhoosith, N and Jaree, A (2021). Biojet fuel production via deoxygenation of crude palm kernel oil using Pt/C as catalyst in a continuous fixed bed reactor. *Energy Conver. Manag.: X*, *12*: 100125.

Mansor, H N; Nazirah, C J; Johari, M A; Parthiban, K; and Tan, S P (2021). Acceptance of good agricultural practices (GAP) among independent oil palm smallholders in Malaysia. *International Journal of Modern Trends in Social Sciences*, *4(16)*: 1-12. DOI: 10.35631/IJMTSS.416001.

Mariam, N M D N S; Hoong, S S; Arniza, M Z; Adnan, S, Ismail, T N M T; Yeong, S K and Yusop, M R (2021). Optimisation of reaction parameters for the synthesis of solketal levulinate as potential biodiesel additive. *J. Oil Palm Res*. DOI: 10.21894/jopr.2021.0052.

Martin, A A; Fodjo, E K; Marc, G B I; Albert, T and Kong, C (2021). Simple and rapid detection of free 3-monochloropropane-1, 2-diol based on cysteine modified silver nanoparticles. *Food Chem.*, *338*: 127787.

Martinez, A; Camberato, J J; Owens, P and Ashtekar, J (2021). Using terrain algorithms on a digital elevation model to evaluate yield variability in oil palm. *J. Oil Palm Res., 33(1)*: 84-92.

Masharuddin, S M S; Karim, Z A A; Said, M A M; Amran, N H and Ismael, M A (2021). The evolution of a single droplet water-in-palm oil derived biodiesel emulsion leading to micro-explosion. *Alex. Eng. J., 61(1)*: 541-547.

MATRADE (2022). Top 10 major export products 2021. https://www.matrade.gov.my/en/malaysian-exporters/services-for-exporters/trade-market-information/trade-statistics/28-malaysian-exporters/trade-statistics/5255-top-10-major-export-products-2021, accessed on 13 February 2022.

Ming, S C; Hung, P K J: Ming, K Y; Aziz, Z F A and Huat, O K (2021). Economic injury level of oil palm bunch moth, *Tirathaba mundella* walker for pest management recommendations in oil palm production. *J. Oil Palm Res., 33(1)*: 37-45.

Mohamaddan, S; Rahman, M A; Andrew Munot, M; Tanjong, S J: Deros, B M: Md Dawal, S Z and Case, K (2021). Investigation of oil palm harvesting tools design and technique on work-related musculoskeletal disorders of the upper body. *Int. J. Ind. Ergon.*, *86*: 103226.

Mohamad, S A; Sharif, M N Y; Ahmad, S N; Masri, M M M; Hung, K J and Kamarudin, N (2021). Population density of *Elaeidobius kamerunicus* FAUST in different spikelet position at anthesising male inflorescence of *Elaeis guineesis* Jacq. in Sabah and Sarawak, Malaysia. *J. Oil Palm Res., 33(1)*: 21-36.

Mohammad, R; Pebrian, D E; Azmi, M A and Husin, E M (2021). Mapping the nitrogen status on immature oil palm area in Malaysian oil palm plantation with autopilot tractor-mounted active light sensor. *J. Oil Palm Res., 33(4)*: 629-642.

MohdAzlan, J and Lok, L (2021). Is there a sustainable future for wildlife in oil palm plantations in Malaysia?. *J. Oil Palm Res., 33(4)*: 732-738.

Mohd Firdaus, O; Rohaya, M H; Miskandar, M S and Astimar, A A (2021). Nutrient enhancement of palm kernel cake via solid state fermentation by locally isolated *Rhizopus oryzae* ME01. *J. Oil Palm Res., 34(1)*: 92-103. DOI: 10.21894/jopr.2021.0022.

Mohd, N K; Lim, W H; Armylisas, A H N and Yeong, S K (2021a). Potential application of palm oil products as electrical insulating medium in oil-immersed transformer. *Environ. Prog. Sustain. Energy*, *40*: e13728. DOI: 10.1002/ep.13728.

Mohd, N K; Yeong, S K; Ibrahim, N A; Nor, S M M; Yunus, W M Z W; Ghazali, M J and Lim, W H (2021b). Corrosion inhibition, adsorption and thermodynamic properties of hydrophobic-tailed imines on carbon steel in hydrochloric acid solution: a comparative study. *J. Adhes. Sci. Technol.*, *35*: 2558-2579. DOI: 10.1080/01694243.2021.1892426.

Mos, H; Harun, M H; Khoon, K H; Jantan, N M; Razak, A A; Shahabuddin, N A; Othman, H; Kamarudin, N; Parveez, G K A; Din, A K; Selliah, P, Salam, N D and Yusup Y (2021). Soil carbon dioxide (CO_2) efflux rate and oil palm yield from different peat types in Sarawak, Malaysia. *J. Oil Palm Res., 33(2)*: 257-266.

MPIC (2018). *The employment survey in oil palm plantations, Malaysia 2018*. Ministry of Plantation Industries and Commodities, Putrajaya.

MPOB (2022a). *Malaysian Oil Palm Statistics 2021*. 41st Edition. MPOB, Bangi.

MPOB (2022b). *Review of the Malaysian Oil Palm Industry 2021*. MPOB, Bangi.

MPOB (2021). *Report on Labour Situation in the Malaysian Oil Palm Plantation Sector*. Internal MPOB Quarterly Reports. Unpublished.

MPOB (2020). *Report on Labour Situation in the Malaysian Oil Palm Plantation Sector*. Internal MPOB Quarterly Reports. Unpublished.

MPOB (2019). *Report on Labour Situation in the Malaysian Oil Palm Plantation Sector*. Internal MPOB Quarterly Reports. Unpublished.

Muanruksa, P; Wongsirichot, P; Winterburn, J and Kaewkannetra, P (2021). Valorization of palm oil soap stock waste toward for biodiesel production: Process optimization under zero waste concept. *Biomass Bioenergy*, *154*: 106231.

Myint, K A; Zulkifli, Y; Rafii, M Y; Oladosu, Y; Abd Samad, M Y; Ramlee, S I; Mustaffa, S; Arolu, F; Abdullah, N; Marjuni, M and Mohd Din (2021). A genetic diversity assessment of MPOB-Senegal oil palm germplasm using microsatellite markers. *BioMed Res. Int*. DOI: 10.1155/2021/6620645.

Nadzirah, A; Chan, P L; Chan, K L; Ong, P W; Morris, P E; Ong-Abdullah, M; Subhi, S M and Low, E T L (2022). Determination of reliable reference genes for reverse transcription quantitative real-time PCR from oil palm transcriptomes. *J. Oil Palm Res.* DOI: 10.21894/jopr.2022.0002.

Nagapan, G; Goh, Y M; Dian, N L H M; Idris, C A C; Selvaduray, K R and Balasundram, N (2021). Effects of interesterified fats on lipoprotein sub fractions and hepatic gene expressions in a hamster model. *J. Oil Palm Res.* DOI: 10.21894/jopr.2021.0046.

Nagappan, J; Kadri, F; Chin, C F; Cooper, R M; May, S T; Abu Seman, I and Low, E T L (2021). A rapid and efficient DNA extraction protocol for *Ganoderma zonatum*, a basal and upper stem rot pathogen of oil palm in Malaysia. *J. Oil Palm Res., 33(1)*: 12-20. DOI: 10.21894/jopr.2020.0058.

Nasrin, A B; Loh, S K; Sukiran, M A; Bukhari, N A; Aziz, A A; Lim, J; Lim, S and Chin, E (2021). Production and characterization of low-ash empty fruit bunches pellets as a solid biofuel. *BioEnergy Res.*, 15: 517-529. DOI: 10.1007/s12155-021-10316-x.

Nazirah C J; Humaira, M T; Zaki, A; Sohimi M R., and Ainul, S S (2021). Workforces among oil palm independent smallholders (OPISH). *Journal of Oil Palm and Palm Oil Research*, *2(2)*: 91-105.

Ng, W Z: Obon, A A; Lee, C L; Ong, Y H; Gourich, W; Maran, K; Tang, D B Y; Song, C P and Chan, E S (2021). Techno-economic analysis of enzymatic biodiesel co-produced in palm oil mills from sludge palm oil for improving renewable energy access in rural areas. *Energy*, *243*: 122745. DOI: 10.1016/j.energy.2021.122745.

Noor, M H M; Lee, W J; Azli, M F Z M; Ngadi, N; Mohamed, M; Inuwa, I M and Opotu, L A (2021a). Microwave- *vs.* ultrasonic-synthesis of magnetic Moringa oleifera coagulant for the reduction of chemical oxygen demand in palm oil wastewater. *Environ. Technol. Innov.*, *24*: 102069.

Noor, N M; Ismail, T N M T; Noor, M A M; Palam, K D P; Sattar, M N; Hanzah, N A; Adnan, S and Yeong, S K (2021b). Physicochemical properties of palm olein-based polyols prepared using homogeneous and heterogeneous catalysts. *J. Oil Palm Res., 34(1)*: 167-176. DOI:10.21894/jopr.2021.0034.

Norizan, M S; Wayayok, A; Karim, Y A; Abdullah, A F and Mahadi, M R (2021). Quantitative approach for irrigation requirement of oil palm: Case study in Chuping, Northern Malaysia. *J. Oil Palm Res., 33(2)*: 278-288.

Nur Azreena, I; Lau, H L N; Asikin-Mijan, N; Hassan, M A; Izham, S M; Kennedy, E; Stockenhuber, M; Mastuli, M S; Alharthi, F A; Alghamdi, A A and Taufiq-Yap, Y H (2021). A promoter effect on hydrodeoxygenation reactions of oleic acid by zeolite beta catalysts. *J. Anal. Appl. Pyrolysis*, *155*: 105044.

Nur, H B; Parthiban, K; Khairuman, H; Johari, M A; Nursuhana, D; and Mohd, K A I (2021). Acceptance level of independent oil palm smallholders in Malaysia towards extension services. *International Journal of Modern Trends in Social Sciences*, *4(18)*: 17-33. DOI: 10.35631/IJMTSS.418003.

Oil World (2021). *Oil World Monthly*. *No. 50*, *Vol. 64*. 17 December 2021. ISTA Mielke GmbH, Germany.

Oil World (2022). *Oil World Weekly Statistics Update*. 1 April 2022. ISTA Mielke GmbH, Germany.

Ooi, L C L; Low, E T L; Ong-Abdullah, M; Rajanaidu, N; Ting, N C; Nagappan, J; Abd Manaf, M A; Chan, K L; Halim, M A; Azizi, N; Omar, W; Murad, A J; Lakey, N; Ordway, J M; Favello, A; Budiman, M A; Van Brunt, A; Beil, M; Leininger, M T; Jiang, N; Smith, S W; Brown, C R; Kuek, A C S; Bahrain, S; Hoynes-O'Connor, A; Nguyen, A Y; Chaudhari, H G; Shah, S A; Choo, Y M; Sambanthamurthi, R and Singh, R (2016). Non-*tenera* contamination and the economic impact of *SHELL* genetic testing in the Malaysian independent oil palm industry. *Front. Plant Sci.*, *7*: 771. DOI: 10.3389/fpls.2016.00771.

Ooi, S E; Feshah, I; Azimi, N; Sarpan, N; Nabeel, A; Lim, C C; Choo, C N; Wong, W C; Wong, F H; Wong, C K and Ong-Abdullah, M (2021). Leaf transcriptomic signatures for somatic embryogenesis potential of *Elaeis guineensis*. *Plant Cell Rep.*, *40*: 1141-1154. DOI: 10.1007/s00299-021-02698-1.

Our World in Data (2021). Forests and deforestation. https://ourworldindata.org/forests-and-deforestation, accessed on 12 February 2022.

Panarmasar, N; Hinchiranan, N and Kuchonthara, P (2021). Catalytic hydrotreating of palm oil for bio-jet fuel production over Ni supported on mesoporous zeolite. *Materials Today: Proceedings*. DOI: 10.1016/j.matpr.2021.09.385.

Parsons, S; Raikova, S and Chuck, C J (2020). The viability and desirability of replacing palm oil. *Nat. Sustain.*, *3*: 412-418. DOI: 10.1038/s41893-020-0487-8.

Parveez, G K A; Ahmad Tarmizi, A H; Sundram, S; Loh, S K; Ong-Abdullah, M; Poo Palam, K D; Salleh, K M; Ishak, S M and Idris, Z (2021). Oil palm economic performance in Malaysia and R&D progress in 2020. *J. Oil Palm Res., 33(2)*: 181-214.

Parveez, G K A; Hishamuddin, E; Loh, S K; Ong-Abdullah, M; Salleh, K M; Bidin, M N I Z; Sundram, S; Hasan, Z A A and Idris, Z (2020). Oil palm economic performance in Malaysia and R&D progress in 2019. *J. Oil Palm Res., 32(2)*: 159-190.

Phichitsurathaworn, N; Simasatitkul, L; Amornraksa, S; Anantpinijwatna, A; Charoensuppanimit, P and Assabumrungrat, S (2021). Techno-economic analysis of co-production of bio-hydrogenated diesel from palm oil and methanol. *Energy Convers. Manag.*, *244*: 114464.

Poopalam, K D; Raghunanan, L; Bouzidi, L; Yeong, S K and Narine, S S (2021). Aliphatic fatty diamide as renewable phase change materials for thermal energy storage. *J. Energy Storage*, *42*: 102934. DOI: 10.1016/j.est.2021.102934.

Purba, E and Sipayung, R (2021). Confirmation and control of glyphosate-resistant biotype of goosegrass (*Eleusine indica* L.) in Sumatran oil palm. *J. Saudi Soc. Agric. Sci*. DOI: 10.1016/j.jssas.2021.10.010.

Rahimi, M S (2021). *Management of Sustainable Palm Oil: Policies and Environmental Considertaions.* 1st edition. UKM, Bangi. p. 41-54.

Rahman, N; Giller, K E, de Neergaard A; Magid, J, de Ven, G V and Bruun, T B (2021). The effects of management practices on soil organic carbon stocks of oil palm plantations in Sumatra, Indonesia. *J. Environ. Manag.*, *278*: 111446.

Rajanaidu, N; Kushairi, A and Mohd Din, A (2017). *Monograph Oil Palm Genetic Resources*. MPOB, Bangi. 289 pp.

Ramli, N A S; Noor, M A M and Ismail, T N M T (2021). Determination of sodium and potassium contents in palm-based polyols using graphite furnace atomic absorption spectrometer. *Chem. Pap.*, *75*: 2561-2574. DOI: 10.1007/s11696-020-01493-8.

Ratnasari, A; Syafiuddin, A; Boopathy, R; Malik, S; Mehmood, M A; Amalia, R; Prastyo, D D and Zaidi, N S (2022). Advances in pretreatment technology for handling the palm oil mill effluent: Challenges and prospects. *Bioresour. Technol.*, *344(B)*: 126239.

Rizali, A; Karindah, S; Nugroho, C T and Rahardjo, B T (2021). Similarity of ant communities increases with isolation from natural habitat and abundance of invasive ants in oil palm plantations of Central Borneo. *Glob. Ecol. Conserv.*, *28*: e01690.

Rodrigues Neto, J C; Vieira, L R; de Aquino Ribeiro, J A; de Sousa, C A F; Souza Júnior, M T and Abdelnur, P V (2021). Metabolic effect of drought stress on the leaves of young oil palm (*Elaeis guineensis*) plants using UHPLC–MS and multivariate analysis. *Sci. Rep.*, *11*: 18271. DOI: 10.1038/s41598-021-97835-x.

Rodriguez, A C; D, Stefano; Schindler, K and Wegner, J D (2021). Mapping oil palm density at country scale: An active learning approach. *Remote Sensing of Environment*, *261*: 112479.

RSPO (2015). *A Shared Vision 100% Sustainable Palm Oil in Europe: A Snapshot of National Initiatives.* RSPO, Kuala Lumpur, Malaysia. 16 pp.

Rudolf, K; Hennings, N; Dippold, M A; Edison, E and Wollni M (2021), Improving economic and environmental outcomes in oil palm smallholdings: The relationship between mulching, soil properties and yields. *Agric. Syst.*, *193*: 103242.

Sahu, J N; Karri, R R and Jayakumar, N S (2021). Improvement in phenol adsorption capacity on eco-friendly biosorbent derived from waste palm-oil shells using optimized parametric modelling of isotherms and kinetics by differential evolution. *Ind. Crops Prod.*, *164*: 113333.

Salih, N and Salimon, J (2021). A review of eco-friendly green biolubricants from renewable and sustainable plant oil sources. *Biointerface Res. Appl. Chem.*, *11*: 13303-13327. DOI: 10.33263/BRIAC115.1330313327.

Salimon, J; Salih, N and Yousif, E (2012). Industrial development and applications of plant oils and their biobased oleochemicals. *Arab. J. Chem.*, *5(2)*: 135-145.

Saminathan, M; Wan Mohamed, W N; Md Noh, A; Ibrahim, N A; Fuat, M A; Kumari Ramiah, S; Lim T C E and Dian, N L H M (2021). Treated oil palm frond and its utilisation as an improved feedstuff for ruminants: An overview. *J. Oil Palm Res*. DOI: 10.21894/jopr.2021.0041.

Sarimana, U; Herrero, J; Pratiwi Erika, P; Indarto, N; Fahmi Wendra, F; Baitha Santika, B; Ritter, E; Sembiring, Z and Dwi Asmono, D (2021). Analysis of genetic diversity and discrimination of Oil Palm DxP populations based on the origins of pisifera elite parents. *Breed. Sci.*, *71(2)*: 134-143. DOI: 10.1270/jsbbs.20043.

Saritpongteeraka, K; Natisupacheevin, K; Tan, C; Rehman, S; Charnnok, B; Vaurs, L P; Leu, S-Y and Chaiprapat, S (2021). Comparative assessment between hydrothermal treatment and anaerobic digestion as fuel pretreatment for industrial conversion of oil palm empty fruit bunch to methane and electricity - A preparation study to full scale. *Fuel*, *310(C)*: 122479. DOI: 10.1016/j.fuel.2021.122479

Sarker, I H (2021). Data science and analytics: An overview from data-driven smart computing, decision-making and applications perspective. *SN Comp. Sci.*, *2*: 377. DOI: 10.1007/s42979-021-00765-8.

Sarpan, N, Tatarinova, T V, Low, E-T L, Ong-Abdullah, M; Sapian, I S and Ooi, S E (2021). Histone modification marks improve identification of oil palm transcription start sites. *J. Oil Palm Res., 34(1)*: 35-45. DOI: 10.21894/jopr.2021.0021.

Sayed, K; Baloo, L; Kutty, S R B M and Makba, F (2021). Potential biodegradation of Tapis light crude petroleum oil, using palm oil mill effluent final discharge as biostimulant for isolated halotolerant *Bacillus* strains. *Mar. Pollut. Bull.*, *172*: 112863.

Seyum, E G; Bille, N H; Abtew, W G; Ntsomboh-Ntsefong, G and Bell, J M (2021). Genome mapping to enhance efficient marker-assisted selection and breeding of the oil palm (*Elaeis guineensis* Jacq.). *Adv. Biosci. Biotechnol.*, *12*: 407-425.

Shaari, N A; Ahmad Tarmizi, A H and Md Sikin, A (2021). In-house validation of accelerated solvent extraction-gas chromatography-mass spectrometry for the determination of bound 3-and 2-monochloropropanediols (MCPD) and glycidol in food products. *Food Addit. Contam. Part A, Chem. Anal. Control Expo. Risk Assess. 38(2)*: 223-236.

Shafirul, A W; Kamil, A T; Ayatollah, K. A. R and Humaira, M T (2021). The adaptability of smallholders toward livestock integration with oil palm and income generated. *Jurnal Penyelidikan Sains Sosial*, *4(12)*: 67-78.

Shahrul, M N K; Abdullah, S N A; Mas Muniroh, M N; Moradpour, M; Noor Azmi, S and Ong-Abdullah, M (2021). Single nucleotide polymorphisms in oil palm homogentisate geranyl-geranyl transferase promoter for species differentiation and *Tocotrienol* improvement. *Meta Gene.*, *27*: 100818. DOI: 10.1016/j.mgene.2020.100818.

Show, K Y; Lo, E K V; Wong, W S; Lee, J Y; Yan, Y and Lee, D J (2021). Integrated anaerobic/oxic/oxic treatment for high strength palm oil mill effluent. *Bioresour. Technol.*, *338*: 125509.

Shulaev, V; Cortes, D; Miller, G and Mittler, R (2008). Metabolomics for plant stress response. *Physiol. Plant.*, *132*: 199-208.

Siddiqui, Y; Surendran, A; Paterson, R R M; Ali, A and Ahmad, K (2021). Current strategies and perspectives in detection and control of basal stem rot of oil palm. *Saudi J. Biol. Sci.*, *28*: 2840-2849.

Siliskisoaps (2022). https://www.siliskisoaps.com, accessed on 17 May 2022.

Singh, R; Low, E T L; Ooi, L C L**;** Ong-Abdullah, M; Ting, N C; Nagappan, J; Rajanaidu, N; Mohd Din, A; Rosli, R; Abdul Manaf, M A; Chan, K L; Halim, M A; Azizi, N; Lakey, N; Smith, S W; Budiman, M A; Hogan, M; Bacher, B; Van Brunt, A; Wang, C; Ordway, J M; Sambanthamurthi, R and Martienssen, R A (2013). The oil palm *SHELL* gene controls oil yield and encodes a homologue of SEEDSTICK**.** *Nature*, *500*: 340-344.

Singh, R; Lee, K T; Ooi, L C L; Low, E T L; Ong-Abdullah, M; Sambanthamurthi, R and Azman, I (2021a). An overview of the development of the oil palm industry and impact of the shell gene innovation as a quality control tool to improve productivity. *J. Oil Palm Res*. DOI: 10.21894/jopr.2021.0001.

Singh, R; Ooi, L C L; Ting, N C; Low, E T L; Ong-Abdullah, M; Rajanaidu, N; Marjuni, M; Mustaffa, S; Abdul Manaf, M A; Garner, P; Reed, J; Wischmeyer, C; Beil, M; Bacher, B; Lakey, N D; Ordway, J M and Sambanthamurthi, R (2021b). A genetic platform for predicting and reducing non-*tenera* contamination in oil palm (*Elaeis guineensis*) seed supply. *Tree Genet. Genomes*, *17*: 45. DOI: 10.1007/s11295-021-01527-6.

Sohimi, M R; Humaira, M T; Zaki, A; Mohd; H A H; and Ainul; S S (2021). Implementation of crop integration with oil palm under government assistance scheme by oil palm independent smallholders (OPISH) in Malaysia. *Jurnal Penyelidikan Sains Sosial*, *4(12)*: 22-30.

Subramaniam, S; Anandha Rao, J S; Ramdas, P; Ng, M H; Kannan Kutty, M; Selvaduray, K R and Radhakrishnan, A K (2021a). Palm gamma-tocotrienol supplementation suppress tumour growth and metastasis in a syngeneic mouse model of breast cancer. *J. Oil Palm Res*. DOI: 10.21894/jopr.2021.0048.

Subramaniam, S; Anandha Rao, J S; Ramdas, P; Ng, M H; Kannan Kutty, M; Selvaduray, K R and Radhakrishnan, A K (2021b). Reduced infiltration of regulatory T cells in tumours from mice fed daily with gamma-tocotrienol supplementation. *Clin. Exp. Immunol.*, *206(2)*: 161-172.

Subramaniam, V; Loh, S K and Aziz, A A (2021). GHG analysis of the production of crude palm oil considering the conversion of agricultural wastes to by-products. *Sustain. Prod. Consum.*, *28*: 1552-1564.

Suharjito, Elwirehardja, G N and Prayoga, J S (2021). Oil palm fresh fruit bunch ripeness classification on mobile devices using deep learning approaches. *Computers and Electronics in Agriculture*, *188*: 106359.

Sulaiman, M R; Mohamad, S A; Masri, M M M; Ahmad, S N and Kamarudin, N (2021a). Preliminary study on efficiency of different time and light source in light traps for capturing population of adult oil palm bunch moth, *Tirathaba mundella* (Lepidoptera: Pyralidae). *J. Oil Palm Res., 33(3)*: 413-424.

Sulaiman N; Yeoh, C B; Khairuddin, N S K and Ahmad Bustamam, F K (2021b). Development and validation of an LCMS/MS method for determination of residual 2,4-dichlorophenoxyacetic acid herbicide in palm oil. *J. Oil Palm Res*. DOI: 10.21894/jopr.2021.0042.

Tang, S W; Chan, C H; Mohd, N K; Lim, W H; Hoong, S S; Ismail, T N M T and Yeong, S K (2021). Synthesis and physicochemical properties of low viscosity 2-ethylhexyl alkyl ethers made from palm-based esters as potential biolubricant. *Eur. J. Lipid Sci. Technol.*, *124*: 2100072. DOI: 10.1002/ejlt.202100072.

Tan, P Y and Teng, K T (2021). Role of dietary fat on obesity-related postmenopausal breast cancer: Insights from mouse models and methodological considerations. *Breast Cancer*, *28(3)*: 556-571.

Tan, Y Y; Bello, M M and Raman, A A A (2021). Towards cleaner production in palm oil industry: Advanced treatment of biologically-treated POME using palm kernel shell-based adsorbent. *Cleaner Eng. Technol.*, *2*: 100079.

Tapia, J F D; Doliente, S S and Samsatli, S (2021). How much land is available for sustainable palm oil. *Land Use Policy*, *102*: 105187.

Tay, B Y P and Wai, W H (2021). A gas chromatography-mass spectrometry method for the detection of chlorpyrifos contamination in palm-based fatty acids. *J. Am. Oil Chem. Soc.*, *98*: 881-887.

Tezara, W; Domínguez, T S T; Loyaga, D W; Ortiz, R N; Chila, V H R and Ortega, M J B (2021), Photosynthetic activity of oil palm (*Elaeis guineensis*) and interspecific hybrid genotypes (*Elaeis oleifera* × *Elaeis guineensis*), and response of hybrids to water deficit. *Sci. Hortic.*, *287*: 110263.

Tivanello, R G; Capristo, M F; Leme, F M; Ferrari, R A; Sampaio, K A; Arisseto, A P and Vicente, E (2021). Mitigation studies based on the contribution of chlorides and acids to the formation of 3-MCPD, 2-MCPD, and glycidyl esters in palm oil. *ACS Food Sci. Technol.*, *1(7)*: 1190-1197.

Tjaronge, M W and Caronge, M A (2021). Physico-mechanical and thermal performances of eco-friendly fired clay bricks incorporating palm oil fuel ash. *Materialia*, *17*: 101130.

Tupaz-Vera, A; Ayala-Diaz, I; Barrera, C F and Romero, H M. (2021). Selection of elite *dura*-type parents to produce dwarf progenies of *Elaeis guineensis* using genetic parameters. *Agronomy*, *11*: 2581. DOI: 10.3390/agronomy11122581.

Uke, A; Nagaoka, E N; Chuah J A; Zain, N A A; Amir, H G; Sudesh, K; Abidin, N Z H A Z; Hashim, Z and Kosugi, A (2021). Effect of decomposing oil palm trunk fibers on plant growth and soil microbial community composition. *J. Environ. Manag.*, *295*: 113050.

Vargas, L H G; Neto, J C R; de Aquino Ribeiro, J A; Ricci-Silva, M E; Souza, M T Jr; Rodrigues, C M; de Oliveira, A E and Abdelnur, P V (2016). Metabolomics analysis of oil palm (*Elaeis guineensis*) leaf: evaluation of sample preparation steps using UHPLC–MS/MS. *Metabolomics*, *12*: 153. DOI 10.1007/s11306-016-1100-z.

Vijay, V; Pimm, S L; Jenkins, C N and Smith, S J (2016). The impacts of oil palm on recent deforestation and biodiversity loss. *PLoS One*, *11(7)*: e0159668. DOI: 10.1371/journal.pone.0159668.

Voon, P T; Toh, S W H; Ng, T K W; Lee, V K M; Yong, X S; Ng, Y T and Nesaretnam, K (2021a). Is palm mid fraction a healthier choice as a cocoa butter equivalent? *J. Oil Palm Res., 33(3)*: 532-541.

Voon, P T; Toh, S W H; Ng, T K W; Lee, V K M; Yong, X S; Yap, S Y and Nesaretnam, K (2021b). Does palm mid fraction affect adult satiety? *J. Oil Palm Res., 33(4)*: 713-723.

Wae-hayee, M; Pakdeechot, S; Hanifarianty, S and Suksuwan, W (2022). Minimizing water consumption in oil palm sterilization using direct steaming: Effects of sterilization pressure and time. *J. Food Eng.*, *315*: 110804.

Wan Nur Syuhada, W N; Rasid, O A and Parveez, G K A (2021). Molecular cloning of *Elaeis guineensis* phytoene synthase (*Egpsy*) and its expression in mesocarp tissues. *J. Oil Palm Res., 33(3)*: 447-457. DOI: 10.21894/jopr.2020.0113.

Wong, Sharron; Muharam, F M; Rashed, O and Ahmad, K (2021). Soil fungal composition and diversity in oil palm plantation at Sungai Asap, Sarawak, Malaysia. *J. Oil Palm Res., 33(2)*: 215-226.

Yaman, Y T; Bolat, G; Saygin, T B and Abaci, S (2021). Molecularly imprinted label-free sensor platform for impedimetric detection of 3-monochloropropane-1, 2- diol. *Sens. Actuators B Chem.*, *328*: 128986.

Yap, C C; Chan, Y J; Loh, S K; Supramaniam, C V; Soh, A C; Chong, M F and Lim, L K (2021a). Pilot-scale investigation of the integrated anaerobic-aerobic bioreactor (IAAB) treating palm oil mill effluent (POME): startup and performance evaluation. *Ind. Eng. Chem. Res.*, *60(10)*: 3839-3859.

Yap, C C; Loh, S K; Chan, Y J; Supramaniam, C V; Chong, M F; Soh, A C; Lim, L K and Loo, L S (2021b). Synergistic effect of anaerobic co-digestion of palm oil mill effluent (POME) with *Moringa oleifera* extract. *Biomass Bioenergy*, *144*: 105885.

Yap, P; Amiratul, A A A R; Mohamad, H A; Mohd, R I; and Hafifi, H Z (2021c). Factors influencing certification process of Malaysian Sustainable Palm Oil (MSPO) during the preparation phase for

independent smallholders in Malaysia. *Oil Palm Industry Economic J.*, *21(1)*: 13-20.

Yap, S Y; Voon, P T; Cheah, Y K; Lee, V K M and Selvaduray, K R (2021d). Association of dietary fats with gut microbiota profile: How does palm oil fit in? *J. Oil Palm Res*. DOI: 10.21894/jopr.2021.0004.

Yaro, N S A; Napiah, M B; Sutanto, M H; Usman, A and Saeed, S M (2021). Performance evaluation of waste palm oil fiber reinforced stone matrix asphalt mixtures using traditional and sequential mixing processes. *Case Stud. Constr. Mater.*, *15*: e00783. DOI: 10.1016/j.cscm.2021.e00783.

Yeap, W C; Norkhairunnisa, C M K; Norfadzilah, J; Muhammad, R M; Appleton, D R and Harikrishna, K (2021). An efficient clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 mutagenesis system for oil palm (*Elaeis guineensis*). *Front. Plant Sci.*, *12*: 773656. DOI: 10.3389/fpls.2021.773656.

Zheng, J; Fu, H; Li, W; Wu, W; Yu, L; Yuan, S; Tao, W Y W; Pang, T K and Kanniah, K D (2021), Growing status observation for oil palm trees using Unmanned Aerial Vehicle (UAV) images. *ISPRS J. Photogramm. and Remote Sensing*, *173*: 95-121.

Zheng, X; Fu, W; Zheng, K; Gao, B; Lin, L; Liu, W; Lin, Z and Fang, Q (2021). A novel method for the simultaneous determination of esterified 2-/3-MCPD and glycidol in foods by GC-MS/MS. *Food Control*, *123*: 107766.

Zhou, L and Yarra, R (2022). Genome-wide identification and expression analysis of bZIP transcription factors in oil palm (*Elaeis guineensis* Jacq.) under abiotic stress. *Protoplasma*, *259(2)*: 469-483. DOI: 10.1007/s00709-021-01666-6.

Zolkafli, S H; Ting, N C; Nik Mohd Sanusi, N S; Ithnin, M; Mayes, S; Massawe, F; Sambanthamurthi, R; Ismail, I; Zainol Abidin, M I; Roowi, S H; Lee, Y P; Hanafi, N F F and Singh, R (2021). Comparison of quantitative trait loci (QTLs) associated with yield components in two commercial *Dura x Pisifera* breeding crosses. *Euphytica*, *217*: 104. DOI: 10.1007/s10681-021-02825-9.

Zulqarnain, Yusoff, M H M; Ayoub, M; Nazir, M H; Sher, F; Zahid, I and Ameen, M (2021). Solvent extraction and performance analysis of residual palm oil for biodiesel production: Experimental and simulation study. *J. Environ. Chem. Eng.*, *9(4)*: 105519.

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DOI: https://doi.org/10.21894/jopr.2021.0001

AN OVERVIEW OF THE DEVELOPMENT OF THE OIL PALM INDUSTRY AND IMPACT OF THE SHELL GENE INNOVATION AS A QUALITY CONTROL TOOL TO IMPROVE PRODUCTIVITY

SINGH, R[1]; LEE, K T[2]; OOI, L C L[1]; LOW, E T L[1]; ABDULLAH, M O[1]; SAMBANTHAMURTHI, R[1] and AZMAN, I[1]*

ABSTRACT

*The oil palm, from West Africa, has greatly contributed to the economy of Malaysia, especially in raising the living standards of the rural population. The crop has also helped satisfy the growing need for oils and fats by the ever-increasing world population. Nevertheless, the palm oil industry is now at the cross-roads, facing serious challenges in its declining yield brought on by issues such as climate change, shortage of labour and arable land. Modern biotechnology, which can differentiate between high performing and low performing palms in the nursery prior to field planting can help in addressing some of these challenges, especially by improving the crop's productivity. This article relates the historical development of the industry in Malaysia, its contribution to the country's economy and explores how science and technology are necessary for its long-term sustainable development. In line with this, a simple economic model demonstrates the feasibility of applying DNA testing to reduce low yielding non-*tenera *contamination in commercial fields.*

Keywords: biotechnology, oil palm, shell gene.

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INTRODUCTION

The history of oil palm in Southeast Asia harkens back to 1848 when the Dutch brought four seedlings from West Africa to the Bogor Botanical Gardens, Indonesia. They were brought in as ornamentals and not for agriculture (Hunger, 1924; Jagoe 1952). Interestingly, the first oil palm in Malaysia (then Malaya, where Singapore was considered part of Malaya) were those sourced by the Singapore Botanic Gardens in the 1870s from the Royal Botanic Garden in Kew, England (Kushairi *et al.*, 2017). It was not until 1917 that the economic potential of the crop was realised in Malaysia, when Frenchman, Henri Fauconnier, planted the first commercial stand at Tennamaram Estate in Batang Berjuntai, now township Bestari Jaya, Selangor, using seeds from the four Bogor palms in Indonesia (Fauconnier, 1948; Kushairi *et al.*, 2017).

The declining price of rubber, then the major crop in the country, saw the oil palm industry gain momentum in the early 1930s, although there was a hiatus in its expansion in the Second World War (Kushairi *et al.*, 2017). However, from the 1960s the industry took off with the area increasing more than 100-fold from a mere 55 000 ha to 5.74 million hectares in 2016 (Nambiappan *et al.*, 2018). The climate in Southeast Asia (particularly in Malaysia and Indonesia) – having sufficient rainfall which is evenly distributed and good sunshine - is ideal for the crop which produces more than double its yield in its African homeland.

[1] Malaysian Palm Oil Board,
6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.

[2] Business School, Sunway University,
5 Jalan Universiti, Bandar Sunway,
47500 Selangor, Malaysia.

* *Corresponding author e-mail: azman@mpob.gov.my*

HIGH PRODUCTIVITY AND DEMAND FOR EDIBLE OILS FUEL GROWTH OF THE OIL PALM INDUSTRY

Malaysia's attempt to diversify its agriculture (from mainly rubber) in the 1960s was behind the rapid growth of the oil palm industry (Basiron, 2007). Its expansion was aided by the replanting of rubber and other crops with oil palm, as it was considered an extremely profitable crop with oil yield of up to 10 times those of other competing vegetable oil crops (like soybean and rapeseed) per unit land area (Nambiappan *et al.*, 2018). This inherent high productivity is the reason why palm oil now accounts for approximately one third of the oils and fats produced worldwide (Kushairi *et al.*, 2019), despite being planted on merely 5% of the total area dedicated to the production of vegetable edible oils. In fact, being the most productive vegetable oil crop, makes oil palm the ideal choice to meet the increasing demand for oils and fats by the ever-expanding world population, which is expected to exceed 9 billion by 2050 (Béné *et al.*, 2015). The projected rise in the living standards and income of the growing population is also expected to fuel additional requirement for palm oil, as an increase in affluence raises the demand for oils and fats in the daily diet (Murphy, 2014). This is already being observed in some major palm oil importing countries like India, where vegetable oil consumption per capita rose by >90% due to rising income (Murphy, 2007). In fact, Tzachor (2019) predicts that by 2030 a significant proportion of the population (~4.9 billion) will be categorised as middle class, with substantial change in dietary habits, which will likely include increase in the consumption of oils and fats. Currently, Malaysia and Indonesia together account for 85% of the world production of palm oil, making Southeast Asia the most important region in the world to meet the growing demand for edible oils and fats (Alam *et al.*, 2015).

Contribution of Oil Palm to Malaysia's Economic and Social Development

As a developing country, Malaysia sought to improve the economic life of its rural population and agriculture was seen as a key means to this end. For example, resettlement schemes such as the Federal Land Development Authority (FELDA) were established as projects to grant land and farming jobs to the landless poor who were living below the poverty level. The establishment of resettlement schemes was in part also fuelled by the World Bank which saw the potential in Malaysia's agriculture sector to uplift the living standard of rural families (Takata, 2008). The first resettlement schemes planted rubber, but with its falling prices in the 1980s (Barlow, 1997), subsequent schemes relied more heavily on oil palm. The shift from rubber to oil palm demonstrated dynamism in the economic viability and sustainability of replanting schemes, which successfully transformed poor rural agriculture-based settlements into near-urban like towns, with much improved amenities (Barau and Said, 2016). The progressiveness of the resettlement schemes was also evident when by the end of the 1980s opening of new schemes was halted (Sutton, 1989), inadvertently contributing to sustainability practices. Today, smallholders, many in FELDA schemes, account for almost 40% of the oil palm area in the country (Alam *et al.*, 2015) and rely on the crop for their daily livelihood. Cultivation of oil palm by the different sectors in Malaysia is shown in *Table 1*.

TABLE 1. MALAYSIAN OIL PALM AREA (%) BY SECTORS

Sector	2007	2014	2017	2018	2019
Private plantations/ estates	60.3	61.5	61.0	61.0	61.1
Government schemes (FELDA, RISDA, FELCRA)	21.4	17.4	16.1	16.4	16.6
Independent smallholders*	11.0	15.0	16.9	16.8	16.7
State schemes/ Government agencies	7.3	6.1	6.0	5.8	5.6
Total	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**

Note: * Independent smallholders were one of the fastest growing sectors in the industry until 2017.
FELDA - Federal Land Development Authority;
RISDA - Rubber Industry Smallholders Development Authority; FELCRA - Federal Land Consolidation and Rehabilitation Authority.

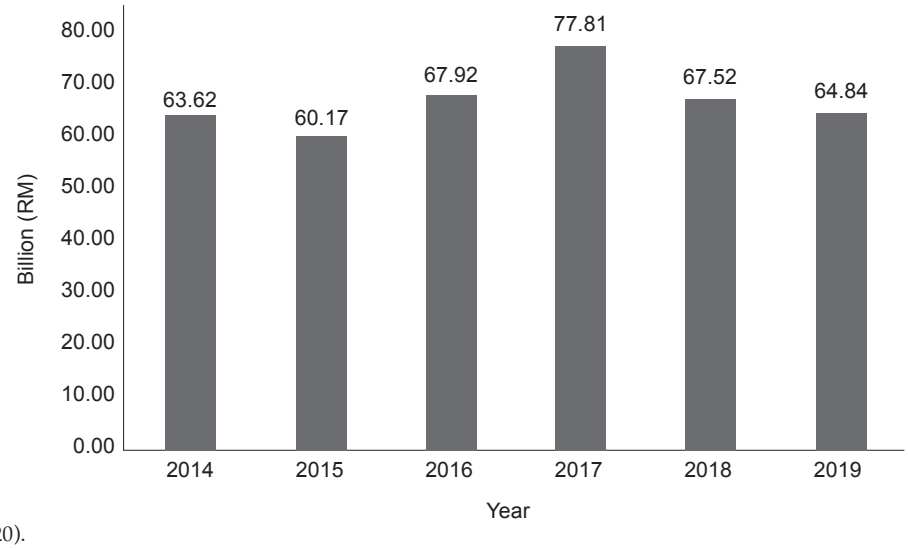
Source: Ab Rahman *et al.* (2008); MPOB (2019); (2020).

The economic success of oil palm in raising the living standards of the rural population is seen in the fact that it contributes 5%-7% to the country's Gross Domestic Product (GDP) (Nambiappan *et al.*, 2018). It is also an important source of employment with over 570 000 employed directly in the industry (Shukoor *et al.*, 2018). With exports of palm oil and its related products exceeding RM60 billion in 2018 (MPOB, 2019), the crop is also a major foreign exchange earner for the country. Despite the occasional hiccups from its low price and low yield due to the weather and

other reasons, export earnings from palm oil and its products have been generally consistent as indicated in *Figure 1*. In fact, the average annual revenue from palm oil and its products over the last six years has been approximately RM66.98 billion (*Figure 1*), a major source of income to the country. The success of the industry lies on the fact that palm oil is highly valued not only as a food, but also has non-food applications, especially in oleochemicals (Chang *et al.*, 2015; Parveez *et al.*, 2015). More recently, palm oil has also gained popularity in the European Union (EU) as a feedstock for biodiesel (Ivancic and Koh, 2016).

Commercial Cultivation of Oil Palm - A Breakthrough in the Belgian Congo Lays the Foundation for Improved Yields

The oil palm that is commercially cultivated today is the *tenera*, which has thin-shell fruit. It is a hybrid from crossing the thick-shell *dura* and shell-less *pisifera* (*Figure 2*). The thin shell of the *tenera* fruit allows it to produce more mesocarp, the oil-bearing tissue, and that is why *tenera* has 30% more oil than *dura* (Corley and Lee, 1992). The shell-less *pisifera*, on the other hand, is usually female sterile, and does not typically produce bunches (Singh *et al.*, 2013a). For this reason, *pisifera* is usually



Source: MPOB (2020).

Figure 1. Export earnings from palm oil and its products (2014-2019).

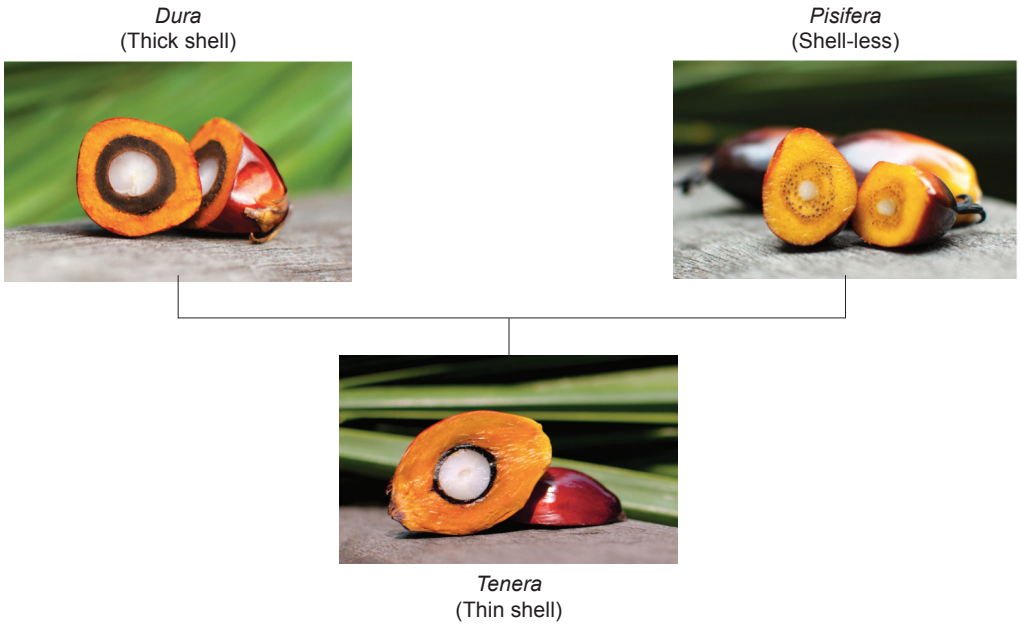


Figure 2. The three fruit forms of oil palm. Tenera *is a hybrid of* dura *and* pisifera *and due to its thinner shell, has more of the orange fleshy oil-bearing mesocarp than* dura *to produce more oil.* Pisifera *is normally female sterile and does not usually produce bunches.*

used as the pollen source (male palm) and *dura* the female parent to produce the hybrid *tenera*.

The early plantations in Malaysia (up to end of 1950s) cultivated pure populations of *dura*, where nearly all palms produced fruit, although they had the less desirable thick shell and thin mesocarp. Interestingly, up to this period some plantations also cultivated palms arising from *dura* x *tenera* crosses (Rajanaidu and Jalani, 1994), which gave a mixture of *dura* and *tenera* palms. However, this was to change due to a discovery made in the 1940s by two European breeders in the Democratic Republic of Congo (then Belgian Congo). These two breeders established that the oil palm has three fruit forms controlled by variants of a single gene, and that crossing the thick-shell *dura* and the shell-less *pisifera* produces palms with the desirable thin-shell *tenera* trait (Beirnaert and Vanderweyen, 1941). Mostly, the fruit of *tenera* was smaller than that of *dura*, less impressive, but with less shell and more mesocarp it actually produced more oil, although it was not established at that time how much more.

This finding was eventually adopted in Malaysian plantations in the 1960s, where the intent from that point forward was to only plant *tenera*. This was also helped by the fact that the new screw press, then recently introduced to express oil from the palm fruits in the 1950s, was more suited to the thin-shell *tenera* fruit (Ornelas, 2000). The planting of *tenera* is considered by many as the 'first wave' of yield improvements that helped the industry gain a strong footing in Malaysia (Davidson, 1993).

Malaysian breeders began to develop and maintain separate female (*dura*) and male (*pisifera*) populations. These breeding materials were further improved by selecting within *dura* x *dura* and *tenera* x *tenera* or *tenera* x *pisifera* crosses. Subsequently, selected *dura* and *pisifera* lines were crossed with the intention to produce seeds of high performing *tenera* palms in commercial fields. The breeders were aware that not all of the *dura* by *pisifera* crosses were 'acceptable' and as such, progeny testing was carried out to determine potentially good parental pairs. In progeny testing trials the offspring were planted and monitored for yield and other performance traits to determine if the parental pairing was suitable for future seed production. This passion for quality control is one of the reasons why Malaysia became a world leader in oil palm cultivation (Basiron, 2007).

Brief Overview of Oil Palm Commercial Seed Production

Commercial cultivation of oil palm begins with seed production from selected *dura* (female) and *pisifera* (male) lines. Currently, there are approximately 24 licensed seed producers in Malaysia, who collectively met the requirement for producing 47.7 million seeds on average from 2016-2019 (MPOB, 2019; 2020). The commercial seeds have to meet the Standards and Industrial Research Institute of Malaysia (SIRIM) standard, MS157 (Rao and Chang, 2018) which is updated regularly. Basically, there are known good palms, or even lines, for both male and female parents. The parental palms have also to satisfy MS157, *i.e.,* they must meet or exceed minimum quality to be used. Pollen from the male palm (*pisifera*) is collected and used to pollinate the female palm (*dura*). The oil palm flowers are borne in inflorescences, and each female flower successfully pollinated will become a seed. The pollinated inflorescence will ripen into a bunch with about 1500-2000 seeds for palms at 10-15 years old (Corley and Tinker, 2008). The pollination is done under strict controlled conditions (Donough *et al.*, 1993) to prevent contamination by stray pollen.

The bunch, on harvest, is brought to the seed laboratory where the seeds are extracted (fruits stripped from the bunch and the mesocarp removed) and stored in refrigerated conditions (Kelanaputra *et al.*, 2018). When an order for the seeds is received, they are subjected to heat treatment to overcome their natural recalcitrance to germination. The whole process from pollination to germination takes about 24 months (Rao and Chang, 2014) without storage. A seed production facility can process up to 100 bunches per day (depending on its capacity) and all seeds from a particular bunch are tagged and kept separately from seeds of other bunches. As such, considerable effort is spent on managing and tracking all samples in a seed production facility. The germinated seeds are planted in polybags in the nursery for 10-12 months before field planting. The palm will bear its first fruit in three years (Singh *et al.*, 2013a). It is only at this time that it is possible to ascertain the fruit type of the palms and assess any contamination of the seed batch by other fruit forms.

Corley (2005) explained how the pollination process can go wrong, and contamination occurs. Some contamination usually occurs (Hama-Ali *et al.*, 2015; Rao *et al.*, 1994) and is acceptable at a low level. The SIRIM standard allows for up to 5% contamination, which acts as a quality guide for oil palm seed producers. However, until recently determining the level of contamination was only possible 4-5 years after purchasing the seeds, when the palms in the field had fruited, by which time it was almost impossible to determine which batch of seeds was contaminated. As such, an assay to differentiate the three fruit forms early, before field planting, would be useful to overcome the contamination. This is just lately available and still in its infancy.

MAJOR RESEARCH AND DEVELOPMENT (R&D) BREAKTHROUGH BY MPOB

A major R&D breakthrough was made by MPOB in 2013, when it decoded the oil palm genome (Singh *et al*., 2013b), and discovered the 'shell gene' and the genetic mechanism of differentiation between the three fruit forms (Singh *et al*., 2013a). This discovery allowed MPOB and Orion BioSains Sdn. Bhd. to develop a simple test to ascertain with near perfect accuracy the fruit form of a palm from its deoxyribonucleic acid (DNA), even from a seed years before its palm fruits (Lakey *et al*., 2017; Low *et al*., 2016; 2018). Thus, planting materials can be tested before field planting for their fidelity.

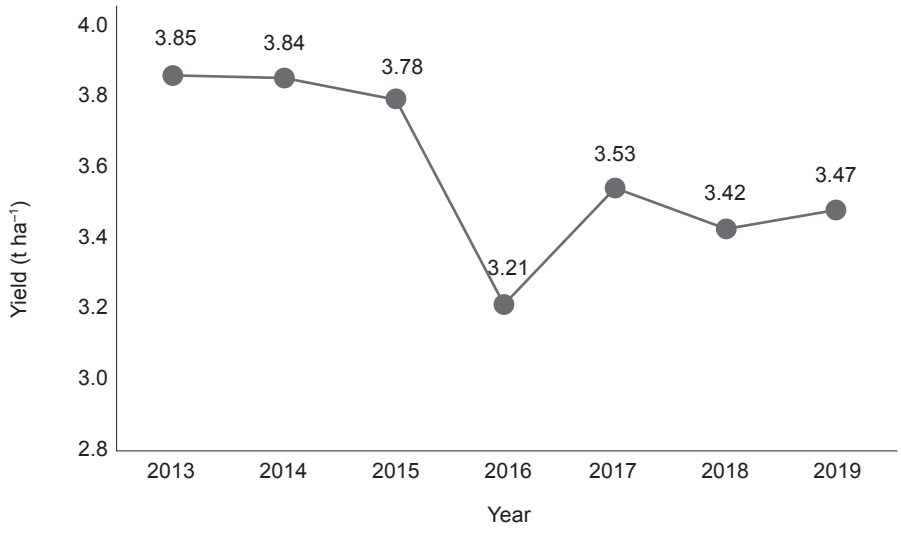
With the DNA test, MPOB and its commercial partner surveyed independent smallholders who normally get their planting materials from nurseries (Ooi *et al*., 2016) for the contamination in their palms. There were earlier reports like, for example, Oberthür *et al.* (2012) of up to 40.0% contamination by typing their mature palms. Ooi *et al.* (2016) used *SHELL* DNA testing to assess 57 sites across all Malaysia, sampling >10 000 palms and found 10.9% contamination, twice the SIRIM standard. Indeed, one nursery sold >60.0% non-*tenera* palms, and 12 sites had >15.0% contaminants, confirming the earlier observation by Oberthür *et al.* (2012). The results were alarming, as it was generally assumed that the contamination of the past had been contained with sufficient quality control measures (Corley, 2005).

Rao *et al.* (1994) and Oberthür *et al.* (2012) estimated that the oil extraction rate (OER) from fresh fruit bunches (FFB) will fall ~0.5% for every 10.0% *dura* contamination rate. In fact, one of the problems plaguing the Malaysian oil palm industry is its yield stagnation or even decline over the last decade (*Figure 3*). Although production has increased, this was due to the expansion in area and not by yield, and planting material contamination may be a contributing factor to this (Lakey *et al*., 2017).

What was clear from Ooi *et al*. (2016) is that non-*tenera* contamination can now be assessed readily, from the palm DNA, even from seedlings. More importantly, the study showed that non-*tenera* contamination was an issue in the industry, although the severity across the different sectors has yet to be investigated (Rao, 2016). The next obvious question is that if the non-*tenera* contamination, observed at 10.9%, is problematic, does the industry need to take action? To answer this question, looking only at the independent smallholder sector (15.0% of oil palm planted area), Ooi *et al.* (2016) did an economic analysis and found that with a 10.9% contamination, the country suffered substantial economic losses of >RM1 billion annually from lower yields in the sector. This suggests that the use of modern biotechnology techniques, such as DNA testing to remove non-*tenera* contamination, can improve the overall industry productivity.

Impact of Drop in Productivity

The importance of having the best planting material, especially for oil palm, is due to its vulnerability to many factors, including weather. In *Figure 3*, it was obvious that in 2016, oil palm suffered a drop in production due to the *El Nino* weather phenomenon, which brought a long period of dry and hot weather in Southeast Asia at the end



Source: MPOB (2020).

Figure 3. Palm oil yield/ha in Malaysia (2013-2019). A general steady decline is observed and contamination of planting material could be a contributory factor. The sharp decline in 2016 was due to an El Nino *that reduced rainfall to stress the palms (Kamil and Omar, 2017).*

of 2015 and early 2016 (Kamil and Omar, 2017). This resulted in a 13.0% drop in crude palm oil (CPO) production in 2016 compared to 2015, causing a shortage with the assumption that demand was unchanged. The supply curve shifted to the left, which resulted in a price increase (*Figure 4*). CPO prices rose to RM2653 t^{-1} on average in 2016, an increase of 23.0% compared to 2015. Although export revenue for the country saw an increase of 7.3% (due to the higher CPO price) (MPOB, 2017), this was a temporary gain, as higher CPO prices result in palm oil being less competitive than other vegetable oils. The vulnerability of the oil palm industry to such factors can be minimised to some extent if biotechnology tools are infused to ensure the best genotypes are identified and planted.

Figure 4. Shortage of palm oil supply in 2016, caused the supply curve (S_1) to shift to the left (S_2). Price increased to RM2653 t^{-1} (Pe_2) from RM2153.50 (Pe_1). Quantity supplied dropped from Qe_1 to Qe_2, to reach equilibrium at point a, with the assumption that demand for palm oil remained unchanged.

Improving Agricultural Productivity with Infusion of Modern Technology

The year 2008 saw a global food crisis (Lusser *et al.*, 2012) that reinforced the need to use modern biotechnology to innovate agriculture. This is even more necessary when the world population is expected to increase to 9.1 billion by 2050, with the projected food production to increase over 70% from 2005-2050 to cater to the added demand (Tomlinson, 2013). Innovation in agriculture is even more critical now that the availability of more land to farm is limited. The way to go is either by producing new and improved varieties or selecting the best materials for planting – to give increased yields and be more tolerant to pests and diseases and resilient to climate change.

Broadly, modern biotechnology will be used to produce better plants, either by genetically engineering them or by assisting conventional breeding using DNA diagnostic tools. Genetically engineered crops are gaining popularity with already an estimated 11% of the world's crops being such (Sandin and Moula, 2015). Some of the major successes are soybean (82% genetically modified - GM), cotton (68% GM), maize (30% GM) and canola (25% GM) (James, 2014). However, there remains opposition to GM foods, especially in the European Union and Africa, mainly on the grounds of human health and the environment (Sandin and Moula, 2015). Basically, the opposition stems from a lack of understanding of the technology and this has led to overly stringent requirements for GM products to be released. Recent developments, especially gene editing to hasten genetic gain, appears less controversial. Nevertheless, the European Court of Justice recently ruled that even the products of gene editing would have to go through the same regulatory process as GM crops, which has dampened enthusiasm for the tool (Urnov *et al.*, 2018).

Oil palm is currently GM-free and palm oil products are spared the regulatory burden of genetically modified organisms. This is, at present, an advantage as the industry can focus on other more pressing issues, such as the environmental concerns in its planting. In order to improve oil palm yield and tackle pests and diseases as well as climate change, the focus is to improve current conventional breeding techniques by using DNA-based diagnostic assays (Sambanthamurthi *et al.*, 2009). The assays basically identify in a collection, the individuals/cultivars/varieties that outperform without the need for extensive field trials, saving time and resources in otherwise ponderous efforts to develop new planting materials (Bevan *et al.*, 2017). This does not require genetically modifying the plant, merely selecting the best from the existing natural variation. It is therefore less controversial as it is basically just fine tuning the selection process that humans have been doing for ages.

However, to apply a DNA-based diagnostic assay, several criteria have to be established. The first is that the specific DNA signal linked to the trait being selected for must first have been scientifically established and validated by independent parties. Accuracy of the assay developed from the association of the trait to a DNA signal is the next important requirement especially in applying it in a commercial setting (Vanderzande *et al.*, 2018). Finally, DNA based selection must be economically feasible for it to be viable in large scale application.

In the case of the oil palm *SHELL* gene, Singh *et al.* (2013a) clearly showed the association of the trait with a gene, which was validated in many independent studies (Babu *et al.*, 2017; Reyes

et al., 2015). Subsequently Lakey *et al.* (2017); Ooi *et al.* (2016) and more recently Singh *et al.* (2020) demonstrated the accuracy of the oil palm DNA assay to differentiate *dura*, *tenera* and *pisifera* palms. The economic losses due to the non-*tenera* palms present in current commercial fields was also predicted by Ooi *et al.* (2016). However, a simple analysis that shows the gains to be obtained in terms of CPO and palm kernel (PK) yield, if DNA testing is implemented to reduce non-*tenera* contamination, will be useful in assessing the feasibility of using DNA testing.

Economic Feasibility of Removing Non-*tenera* Palms Using DNA Assay

This study used the Malaysian national statistics for oil palm in 2017 (MPOB, 2018) to carry out the analysis. The statistics of 2017 were used in this case study, as the oil palm plantings in 2017 will likely more accurately mimic the contamination rates observed by Ooi *et al.* (2016). To evaluate gains from DNA testing, we carried out a two-scenario analysis - the first being that the area planted in 2017 had the level of contamination reported by Ooi *et al.* (2016), that is 8.09% *dura* and 1.30% *pisifera*. The second scenario was a hypothetical situation, where all the palms planted in 2017 had been tested and the contamination rate was brought down to 0.5%. Another assumption made was that since contamination was reduced to as low as 0.5% after testing, it was limited to *dura*, where *pisifera* contamination was removed altogether from the system, as its levels were much lower to start with. Contamination was kept at 0.5% *dura* and not 0.0%, as it was our opinion that any test implemented in a biological system, for whatever reason, will not achieve zero perfection. The difference in the monetary value of CPO and PK production between the two scenarios reflected the financial losses incurred by the industry due to the contamination of non-*tenera* planting materials in commercial fields. The industry statistics used for the economics analysis is summarised below:

Total matured area : 5 110 713 ha
Total CPO production : 19.9 million tonnes
Total PK production : 5 million tonnes
Average CPO price : RM2783 t^{-1}
Average PK price : RM2536 t^{-1}

Source: MPOB (2018).

Although the total area planted in 2017 was recorded as 5.8 million hectares (MPOB 2018), the 5.1 million hectares represents the total mature area, consisting of oil palm producing CPO and PK. *Table 2* below further summarises the total mature area planted with the three different fruit forms, adjusted for *dura* and *pisifera* contamination, and their respective CPO and PK yields.

It was presumed that 90.61% of the area (4.6 million hectares) in 2017 was planted with *tenera* palms while the remainder with *dura* (8.09%, 0.4 million hectares) and *pisifera* (1.3%, 0.07 million hectares) palms. Only 98.7% of the matured area, (minus the 1.3% *pisifera*) totalling 5 044 274 ha produced the 19.9 million tonnes of CPO in 2017. The *dura* hectarage should have ideally contributed 1.63 million tonnes of the total CPO yield of 2017, based on the matured area without *pisifera* palms. However, as *dura* only produces 70% of the yield observed in *tenera* palms, the hectarage in actual fact only contributed 1.1 million tonnes, while the remainder 18.8 million tonnes were likely contributed by the area planted with *tenera* palms. The total CPO yield for the area planted with *tenera* and *dura* also allowed the estimation of the yield/ha for both fruit forms, where the difference, as expected, was close to 70%. Total PK yield is usually a quarter of the CPO yield (Van Gelder, 2004).

Based on the above statistics, the CPO and PK produced under the two scenarios outlined above is summarised in *Table 3*. In the first scenario where palms are planted without testing, the CPO and PK produced in 2017 were almost similar to the official statistics reported (MPOB, 2018). Considering the price for CPO and PK in 2017, the total income was approximately RM68 billion. In the hypothetical scenario where testing was carried out and *dura* contamination was reduced to 0.5%, the total income generated increased to RM70.6 billion, a gain of approximately RM2.6 billion yr^{-1}. Removing non-*tenera* contamination using the DNA based assay

TABLE 2. THE ESTIMATED CRUDE PALM OIL (CPO) AND PALM KERNEL (PK) YIELD FOR *Dura*, *Tenera* AND *Pisifera* PALMS IN 2017

	Rate (%)	Area (ha)	CPO yield (million tonnes)	CPO yield t ha^{-1}	PK yield (million tonnes)	PK yield t ha^{-1}
Tenera	90.61	4 630 817	18.8	4.06	4.7	1.01
Dura	8.09	413 457	1.1	2.76	0.3	0.69
Pisifera	1.30	66 439	-	-	-	-
Total	**100.00**	**5 110 713**	**19.9**	**-**	**5.0**	**-**

TABLE 3. SIMPLE ECONOMIC ANALYSIS OF LOSSES SUFFERED BY INDUSTRY DUE TO LOWER CRUDE PALM OIL (CPO) AND PALM KERNEL (PK) PRODUCTION CAUSED BY NON-*Tenera* CONTAMINATION

CPO		PK	
Actual CPO production in 2017	**19. 9 million tonnes**	**Actual PK production in 2017**	**5 million tonnes**
Estimated CPO from *tenera* palms	18. 8 million tonnes	Estimated PK from *tenera* palms	4.7 million tonnes
Estimated CPO from *dura* palms	1.1 million tonnes	Estimated PK from *dura* palms	0.3 million tonnes
Income 1 (A)	**RM55 435 498 173**	**Income 1 (C)**	**RM12 556 637 096**
Tenera area after screening	5 085 159 ha	*Tenera* area after screening	5 085 159 ha
Dura area after screening (0.5%)	25 554 ha	*Dura* area after screening (0.5%)	25 554 ha
CPO production after screening	**20.7 million tonnes**	**PK production after screening**	**5.14 million tonnes**
Estimated CPO from *tenera* palms	20.6 million tonnes	Estimated PK from *tenera* palms	5.13 million tonnes
Estimated CPO from *dura* palms	0.07 million tonnes	Estimated PK from *dura* palms	0.0017 million tonnes
Income 2 (B)	**RM57 629 115 586**	**Income 2 (D)**	**RM13 052 471 527**
Estimated loss (A-B)	**(RM2 193 617 413)**	**Estimated loss (C-D)**	**(RM496 834 431)**

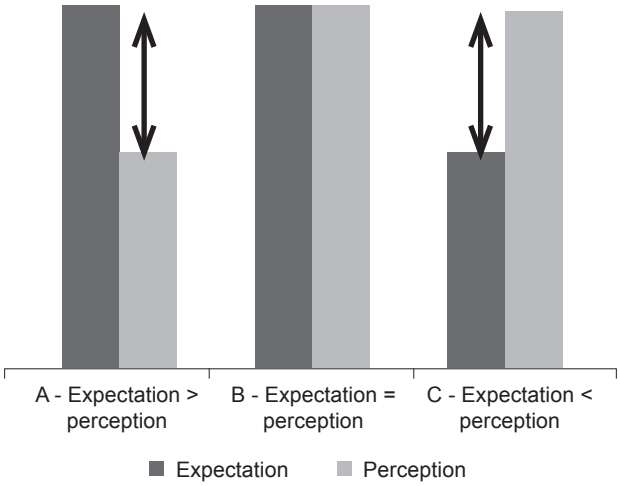
Note: Total gain by removing non-*tenera* contamination = RM2.69 billion.

increases CPO and PK production to more than double that estimated by Ooi *et al.* (2016), which suggests a potentially significant impact on the national economy if implemented by the industry. The increase in profitability will also result in additional tax gains to the Government. The added benefit of DNA testing, is that all agriculture inputs (fertiliser, pest control) are applied on palms that can inherently provide optimal yields. Moving forward, it will be interesting to carry out the analysis by taking into account that the total hectarage consists of palms at different age groups and as such, have varying yield profiles.

DNA and/or protein-based diagnoses are most widely used in medical sciences for the early detection of cancer, especially breast, cervical and colorectal cancer (Krieger *et al.*, 2018; Molparia *et al.*, 2018). DNA-based testing for early detection of cancer or other human diseases is not carried out at present on an entire population, but rather on individuals as recommended by the medical practitioners or by select groups who are aware of and can afford the testing (Foulkes *et al.*, 2016). Although the initial cost of testing and early treatment (if required) can be high (Issa and Noureddine, 2017), the long-term savings to the individual and health care system can be substantial. The use of DNA tests in crops like oil palm has generally lagged human and even animal sciences. Nevertheless, the literature suggests that they are gaining popularity and beginning to be employed widely (Van Nocker and Gardiner, 2014). The most successful application has been in identifying parental lines with certain features (*e.g.*, disease resistance) (Stevanato *et al.*, 2015; Sniezko and Koch, 2017), where the economic justification is obvious. The economic gains appeared dependent on several factors, which include cost of the tests, the number of DNA tests required and severity of culling. Recently Wannemuehler *et al.* (2019) demonstrated that for a perennial crop like apple, making DNA informed decisions that result in culling or removal of at least 12% unwanted materials, can substantially improve the economic efficiency of the apple breeding programme. While the cost of DNA testing nationwide to reduce non-*tenera* contamination has to be determined, improvements in technology are already lowering the cost of DNA-testing (Vanderzande *et al.*, 2018). There is no doubt on the severity of the problem in oil palm as described by Ooi *et al.* (2016), suggesting that severe culling and with it substantial number of DNA tests will be required. Since the *SHELL* DNA test is accurate, the problem is large enough, the economic gains are substantial, it collectively suggests that *SHELL* DNA testing should have good prospects for adoption by the oil palm industry.

Quality Perception of DNA Tested Planting Material

As the producers of oil palm planting materials also sell their product to other companies and independent nurseries, DNA testing will allow their materials to achieve a higher standard (99.5% purity) than the current benchmark set by SIRIM (95.0% purity), resulting in quality perception that will exceed customer expectations as shown in *Figure 5*. If Malaysia takes the lead in implementing DNA testing, the country will establish the bench-mark for quality oil palm planting material globally and help cement Malaysia's role as the world leader in oil palm innovation.



A. Current situation, where non-*tenera* contamination rates exceed Standards and Industrial Research Institute of Malaysia (SIRIM) standards, customer perception of quality of planting materials is below expectation.
B. Customers perception of planting material, is same as expectation if SIRIM standards of 95.0% purity is achieved.
C. If DNA testing implemented, purity of planting materials (99.5% purity) will exceed SIRIM standards (95.0% purity) - customer perception of product will exceed expectation.

Adapted from: Slack *et al.* (2010).

Figure 5. Perceived quality is determined by the gap between customer expectation and perception.

Appropriate Stages to Apply *SHELL* DNA Testing in Oil Palm

Selecting the most appropriate stage to implement DNA testing for making informed decisions will influence its successful implementation. Wannemuehler *et al.* (2020) had demonstrated that incorporating DNA based technology in strawberry was most cost-effective when implemented at the end of seedling trials, instead of at the greenhouse stage. The cost incurred (labour and maintenance) at the greenhouse stage was lower than applying DNA selection at this early stage of strawberry breeding programmes. However, the greenhouse stage was considered the most optimal and cost-effective period to apply DNA based testing in apple, due to its long juvenile phase where the labour and other associated costs are high in conventional programmes, making DNA based selection attractive (Wannemuehler *et al.*, 2019).

Similarly, the cost-effectiveness of testing will be a huge determinant on whether the industry adopts DNA assay for reducing non-*tenera* contamination. As such, any way to lower the cost without compromising the rigour of testing should be identified/adopted (*e.g.*, using seeds or plantlets instead of field palms) for the DNA analysis. To evaluate the best material to carry out the analysis, it is important to assess the whole process in producing commercial planting materials, as summarised in *Figure 6*.

Seeds from individual bunches are kept separate until germination. It is only after germination that the seeds from different batches are mixed for sale. Currently, testing at both the seed and nursery stages is technically possible (Lakey *et al.*, 2017; Low *et al.*, 2018), although nursery testing is more convenient due to easier access. A simple analysis (*Table 4*) demonstrates that testing seeds in the seed production facility is 21% the cost of testing seedlings in the main nursery. The main reason is that a statistical sample of 10% of a seed bunch (or a maximum of 100 seeds/bunch) should be sufficient to predict the contamination rate for the bunch (Lakey *et al.*, 2017; Low *et al.*, 2018). In addition, there will be other substantial savings, as contaminated bunches can be rejected before downstream investments are made in the germination, prenursery and main nursery stages. Furthermore, cleaning up the supply chain earlier also has additional benefits – it is easier to trace the source of problem and rectify it.

Adoption of DNA Testing by the Oil Palm Industry

Economic sustainability requires increase in productivity (yield/ha), lower production costs and stable commodity prices. R&D has been tailored to reduce production cost (*e.g.,* introduction of mechanisation) and increase yields (improved planting material) in order to minimise the effect of fluctuating commodity prices, that are dictated by the free market and beyond the control of the industry. Although the cost of production for oil palm on a per hectare basis is lower than that of soybean and rapeseed (Zimmer, 2016), it is rapidly increasing for oil palm, especially due to rise in fertiliser and labour cost (Wahid and Simeh, 2009). The rise in production cost has to be compensated by the increase in CPO and PK yields/ha. This is possible, among other factors through the utilisation of improved planting material, and also by making sure that only the hybrids (DxP or *tenera*) derived from the selected *dura* and *pisifera* parental material are planted. This also ensures that the inputs that contribute to cost of production (*e.g.*, fertiliser) are only supporting palms that have the genetic potential to produce maximum yield. The presence of non-*tenera* contamination in commercial fields that contributes to significant decline in CPO and PK production suggests that there is a strong basis for the industry to adopt DNA based diagnostic assay to remove undesired palms before field planting.

However, an understanding of how the industry is structured may explain why potential adoption of the technology or other DNA based technologies in the future may face some resistance. Currently,

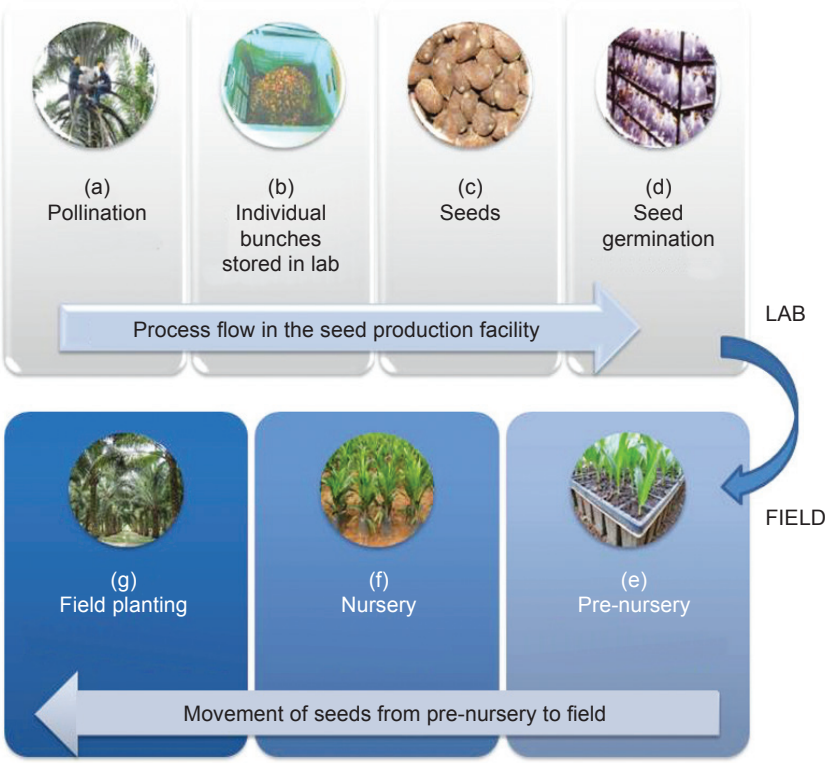


Figure 6. Process flow in commercial oil palm seed production. (a) Pollination; (b) Bunches brought to lab for germination and kept separate; (c) - (d) Upon germination, seeds are individually checked to cull abnormals; (e) - (g) Sowing of germinated seeds in pre-nursery and subsequent movement of seeds from nursery to field. Prior to sowing in the pre-nursery, seeds from the different bunches are kept separate.

TABLE 4. RELATIVE COST ANALYSIS OF SEEDLINGS (in the nursery) OR TESTING SEEDS (at the seed production facility)*

Testing stage	Assumptions	Number of samples tested	Cost of genetic test/sample	Total test cost	Cost of testing per palm for field planting
Leaf testing of seedlings in nursery	i. Testing done after completion of culling exercise	16 100	X	16 100X	X
	ii. 10% extra samples tested to accommodate non-*tenera* contamination				
Bunch by bunch testing of seeds at production facility	i. Each bunch contains 1 000 seeds	Number of bunches: 33	X	3 300X	3 300X/ 15 600X = 21%
	ii. 10% of seeds tested (Lakey *et al.*, 2017)	No. seeds tested: 3 300			(Conclusion: Seed testing is 21% the cost of nursery testing)
	iii. 20% extra bunches tested as 80% bunches pass test (non-*tenera* contamination below 0.5%)	Number of bunches that pass (80%): 26 (26 000 seeds)			
	iv. 40% of seeds either do not germinate, used for testing and are subsequently culled in the nursery	Number that proceed to field planting (60%): 15 600			

Note: *To provide ~14 600 palms to plant a 100 ha area (146 palm ha^{-1}).

less than 10% of the companies engaged in oil palm cultivation in Malaysia are fully integrated, with the relevant upstream (including seed production facility) and downstream activities as illustrated in *Figure 7*.

If non-*tenera* contamination is to be removed, the burden will likely fall on the R&D department, that has to absorb the cost of screening either at the seed production facility or in the nursery, to make sure breeding and commercial fields are free of contamination. However, the benefit of testing will largely be obtained by the downstream sector, which will enjoy higher CPO and PK yields from the lower rates of non-*tenera* contamination. As such, if testing is to be incorporated into the system, commitment is required from higher management for the burden of testing to be shared across all sectors (upstream and downstream) of an integrated company.

Plantation companies that do not have downstream activities, can only accommodate the cost of testing, if mills that accept their FFB are willing to pay the additional cost that guarantees higher CPO and PK yields. Most impacted are independent smallholders who likely will have to pay more for 'DNA tested planting material', with the hope that their FFB can fetch a higher price in anticipation of higher oil yield. Since these smallholders are small scale producers (in terms of FFB), they do not have the capacity to negotiate with dealers or with mills and have to accept the price offered for their produce. As such, an appropriate industry-wide strategy, that builds a strong commitment to obtain the benefits of DNA testing is required. Without a concerted effort and a commitment to change by all stakeholders, DNA testing at present and in the future (DNA testing for other traits) will not receive the same acceptance as seen in other vegetable oil crops.

CONCLUSION

Malaysia has been the world leader in oil palm R&D innovation and together with Indonesia, produces more than 85% of world palm oil. Malaysia's success has been mostly attributed to its superior field and management practices, apart from the excellent R&D efforts, which have been shared with other oil palm growing countries. MPOB's ground-breaking research in oil palm genomics, which led to discovery of the shell gene, which is now developed into a DNA diagnostic tool, paves the way for Malaysia to introduce modern biotechnology approaches to improve oil palm productivity. Since the availability of new land for oil palm cultivation in Malaysia is now limited, and, indeed, may even be shrinking from other uses like urbanisation, the only way forward for the industry is to increase its productivity from the existing area. For this, the need for new biotechnology tools is evident as this study revealed that non-*tenera* contamination (by plants with inferior yields) can be almost two-fold higher than that permissible by SIRIM for oil palm planting materials. As the industry faces serious labour shortage, its quality control in the production of commercial planting materials may be compromised. To arrest the problem, modern biotechnology can be invoked, such as by using the shell gene technology as a quality control tool to ensure the long-term sustainability of the industry. Although the present analysis has clearly demonstrated that adopting DNA testing is financially viable for the industry, the study argues that technological adoption, despite its many benefits, may face resistance from industry. An appropriate industry-wide strategy that facilitates adoption of modern DNA based technologies is thus, required.

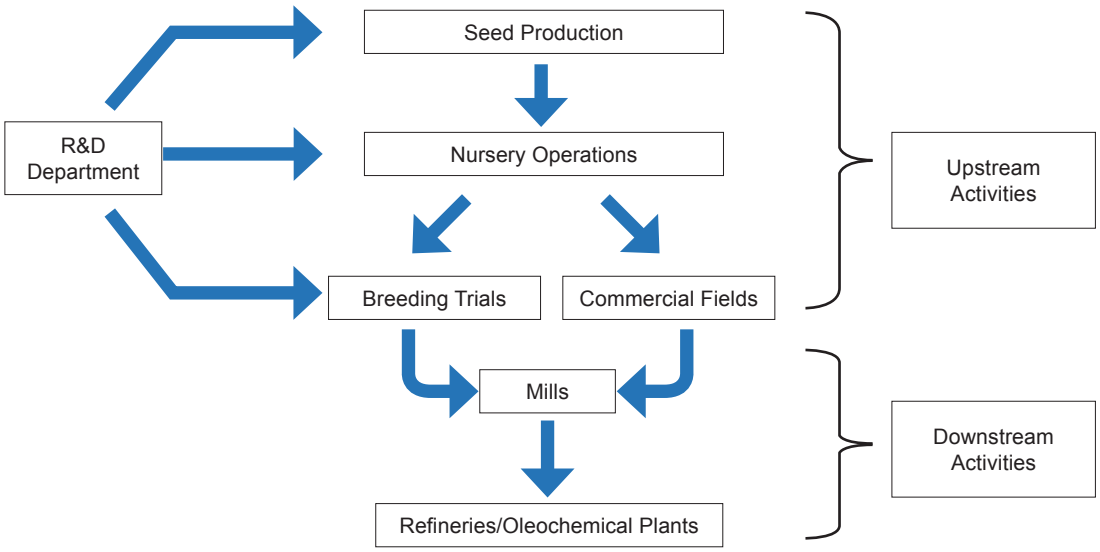


Figure 7. Organisation of upstream and downstream activities in an integrated plantation company.

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REFERENCES

Ab Rahman, A K; Abdullah, R; Mohd Shariff, F and Simeh, M A (2008). The Malaysian palm oil supply chain: The role of the independent smallholders. *Oil Palm Industry Economic J., 8(2)*: 17-27.

Alam, A F; Er, A C and Begum, H (2015). Malaysian oil palm industry: Prospect and problem. *J. Food, Agric. Environ., 13(2)*: 43-148.

Babu, B K; Mathur R K; Kumar, P N; Ramajayam, D; Ravichandran, G; Venu, M V B and Babu, S S (2017). Development, identification and validation of CAPS marker for *SHELL* trait which governs *dura*, *pisifera* and *tenera* fruit forms in oil palm (*Elaeis guineensis* Jacq.). *PLoS ONE, 12(2)*. e0171933

Barau, A S and Said, I (2016). From goodwill to good deals: FELDA land resettlement scheme and the ascendancy of the landless poor in Malaysia. *Land Use Policy, 54*: 423-431.

Barlow, C (1997). Growth, structural change and plantation tree crops: The case of rubber. *World Dev., 25(10)*: 1589-1607.

Basiron, Y (2007). Palm oil production through sustainable plantations. *Eur. J. Lipid Sci. Technol., 109(4)*: 289-295.

Beirnaert, A and Vanderweyen, R (1941). Contribution a l'etude genetique et biometrique des varieties d'*Elaeis guineensis*. *Jacq. Publs. INEAC, Series Ser. Sci, 27*: 101.

Béné, C; Barange, M; Subasinghe, R; Pinstrup-Andersen, P; Merino, G; Hemre, G I and Williams, M (2015). Feeding 9 billion by 2050–Putting fish back on the menu. *Food Sec., 7(2)*: 261-274.

Bevan, M W; Uauy, C; Wulff, B B; Zhou, J; Krasileva, K and Clark, M D (2017). Genomic innovation for crop improvement. *Nature, 543(7645)*: 346-354.

Chang, T S; Yunus, R; Rashid, U; Choong, T S; Biak, D R A and Syam, A M (2015). Palm oil derived trimethylolpropane triesters synthetic lubricants and usage in industrial metalworking fluid. *J. Oleo Sci., 64(2)*: 143-151.

Corley, R H V and Lee, C H (1992). The physiological basis for genetic improvement of oil palm in Malaysia. *Euphytica, 60(3)*: 179-184.

Corley, R (2005). Illegitimacy in oil palm breeding. *J. Oil Palm Res., 17*: 64-69.

Corley, R H V and Tinker, P B (2008). *The Oil Palm*. John Wiley & Sons. p. 47.

Davidson, L (1993). Management for efficient cost effective and productive oil palm plantations. *Proc. of the 1991 PORIM International Palm Oil Conference - Agriculture* (Basiron, Y *et al*., eds.). MPOB, Bangi. p. 153-167.

Donough, C R; Ng, M and Lai, C (1993). Pamol's approach to quality control in controlled pollination for DxP seed production. *Planter, 69*: 163-175.

Fauconnier, H (1948). *The Soul of Malaya*. Penguin, London. 191 pp.

Foulkes, W D; Knoppers, B M and Turnbull, C (2016). Population genetic testing for cancer susceptibility: Founder mutations to genomes. *Nat. Rev. Clin. Oncol., 13(1)*: 41 pp.

Hama-Ali, E O; Alwee, S S R S; Tan, S G; Panandam, J M; Ling, H C; Namasivayam, P and Peng, H B (2015). Illegitimacy and sibship assignments in oil palm (*Elaeis guineensis* Jacq.) half-sib families using single locus DNA microsatellite markers. *Mole. Biol. Rep., 42(5)*: 917-925.

Hunger, F T W (1924). *De Oliepalm* (*Elaeis guineensis*): *Historisch onderzoek over den oliepalm in Nederlandisch-Indie*. N V Boekhandel en Drukkerij voorheen E J Brill, Leiden. 256 pp.

Issa, I A and Noureddine, M (2017). Colorectal cancer screening: An updated review of the available options. *World J. Gastroenterol., 23(28)*: 5086.

Ivancic, H and Koh, L P (2016). Evolution of sustainable palm oil policy in Southeast Asia. *Cogent Environ. Sci., 2(1)*: 1195032.

Jagoe, R B (1952). Deli oil palms and early introductions of *Elaeis guineensis* to Malaya. *Malay Agri. J., 35*: 4-11.

James, C (2014). Preview: Global Status of Commercialized Biotech/GM Crops, ISAAA Brief No. 49. International Service for the Acquisition of Agro-biotech Application, Ithaca, New York.

Kamil, N N and Omar, S F (2017). The impact of *El Niño* and *La Niña* on Malaysian palm oil industry. *Oil Palm Bulletin, 74*: 1-6.

Kelanaputra, E S; Nelson, S P; Setiawati, U; Sitepu, B; Nur, F; Forster, B P and Purba, A R (2018). *Seed Production in Oil Palm: A Manual*. CABI. 75 pp.

Krieger, N; Nabavi, S; Waterman, P D; Achacoso, N S; Acton, L; Schnitt, S J and Habel, L A (2018). Feasibility of analyzing DNA copy number variation in breast cancer tumor specimens from 1950-2010: How old is too old? *Cancer Causes Control, 29(3)*: 305-314.

Kushairi, A; Singh, R and Ong-Abdullah, M (2017). The oil palm industry in Malaysia: Thriving with transformative technologies. *J. Oil Palm Res., 29(4)*: 431-439.

Kushairi, A; Ong-Abdullah, M; Nambiappan, B; Hishamuddin, E; Bidin, M N I Z; Ghazali, R; Subramaniam, V; Sundram, S and Parveez, G K A (2019). Oil palm economic performance in Malaysia and R&D progress in 2018. *J. Oil Palm Res., 31*: 65-194.

Lakey, N D; Ordway, J M; Jiang, N; Smith, S W; Favello, A; Ooi, L C L; Singh, R; Nookiah, R; Low, E T L; Ong-Abdullah, M and Sambanthamurthi, R (2017). Increasing oil palm industry profit, Malaysian gross national income and government tax revenue with breakthrough DNA tests. *Planter, 93(1093)*: 273-279.

Low, E T L; Singh, R; Ong-Abdullah, M; Lakey, N D; Ordway, J M; Smith, S W; Ooi, L C L; Rajanaidu, N; Sambanthamurthi, R; Martienssen, R A and Manaf, M A A (2018). Oil palm genome technology breakthroughs for enhance sustainability of the oil palm industry. *In the 14th International Symposium on Biocatalysis and Agricultural Biotechnology*. 22-24 October 2018. Kuala Lumpur, Malaysia. 103 pp.

Low, E T L; Singh, R; Rajanaidu, N; Ong-Abdullah, M; Ooi, L C L; Lakey, N D; Smith, S W; Ordway, J M and Sambanthamurthi, R (2016). New frontiers for the oil palm industry through genome technology. *Planter, 92(1087)*: 701-710.

Lusser, M; Parisi, C; Plan, D and Rodríguez-Cerezo, E (2012). Deployment of new biotechnologies in plant breeding. *Nat. Biotechnol., 30(3)*: 231-239.

Molparia, B; Oliveira, G; Wagner, J L; Spencer, E G and Torkamani, A (2018). A feasibility study of colorectal cancer diagnosis via circulating tumor DNA derived CNV detection. *PLoS ONE, 13(5)*: p.e0196826.

MPOB (2017). *Malaysian Palm Oil Statistics 2016*. 36th edition. MPOB, Bangi.

MPOB (2018). *Malaysian Palm Oil Statistics 2017*. 37th edition. MPOB, Bangi.

MPOB (2019). *Malaysian Palm Oil Statistics 2018*. 38th edition. MPOB, Bangi.

MPOB (2020). *Malaysian Palm Oil Statistics 2019*. 39th edition. MPOB, Bangi.

Murphy, D J (2007). Future prospects for oil palm in the 21st century: Biological and related challenges. *Eur. J. Lipid Sci. Technol., 109(4)*: 296-306.

Murphy, D J (2014). The future of oil palm as a major global crop: Opportunities and challenges. *J. Oil Palm Res., 26(1)*: 1-24.

Nambiappan, B; Ismail, A; Hashim, N; Ismail, N; Nazrima, S; Idris, N A N; Omar, N; Saleh, K M; Hassan, N A M and Kushairi, A (2018). Malaysia: 100 years of resilient palm oil economic performance. *J. Oil Palm Res., 30(1)*: 13-25.

Oberthür, T; Donough, C R; Indrasuara, K; Dolong, T and Abdurrohim, G (2012). Successful intensification of oil palm plantations with best management practices: Impacts on fresh fruit bunch and oil yield. *Proc. Int'l Planters' Conference*. p. 67-102.

Ornelas, K C (2000). *The Cambridge World History of Food (Vol. 2)*. Cambridge University Press.

Ooi, L C L; Low, E T L; Abdullah, M O; Nookiah, R; Ting, N C; Nagappan, J; Manaf, M A; Chan, K L; Halim, M A; Azizi, N and Omar, W (2016). Non-*tenera* contamination and the economic impact of *SHELL* genetic testing in the Malaysian independent oil palm industry. *Front. Plant Sci., 7*: 771.

Parveez, G K A; Rasid, O A; Masani, M Y A and Sambanthamurthi, R (2015). Biotechnology of oil palm: Strategies towards manipulation of lipid content and composition. *Plant Cell Rep., 34(4)*: 533-543.

Rajanaidu, N and Jalani, B S (1994). Influence of planting material on oil extraction ratio (OER). *National Seminar on Palm Oil Extraction Rate: Problem's and Issues* (Ariffin, D and Jalani, B S eds.). 21-22 December 1994. PORIM, Bangi. p. 53-57.

Rao, V (2016). Oil palm shell gene and DxP purity in independent plantings. *The Planter, 92(1086)*: 663-664.

Rao, V; Jalani, B S and Rajanaidu, N (1994). Effect of *dura* contamination on oil extraction rate (OER). *National Seminar on Palm Oil Extraction Rate: Problems and Issues* (Ariffin, D and Jalani, B S eds.). 21-22 December 1994. PORIM, Bangi. p. 58-60.

Rao, V and Chang, K C (2014). *Oil Palm Seed Production: Field and Laboratory Manual*. MPOB, Bangi. 88 pp.

Rao, V and Chang, K C (2018). MS 157: An evolving story. *The Planter, 94(1109)*: 491-502.

Reyes, P A; Ochoa, J C; Montoya, C; Daza, E; Ayala, I M and Romero, H M (2015). Development and validation of a bi-directional allele-specific PCR tool for differentiation in nurseries of *dura*, *tenera* and *pisifera* oil palms. *Agronomía Colombiana, 33(1)*: 5-10.

Sambanthamurthi, R; Singh, R; Kadir, A P G; Abdullah, M O and Kushairi, A (2009). Opportunities for the oil palm via breeding and biotechnology. *Breeding Plantation Tree Crops: Tropical Species*. Springer, New York. p. 377-421.

Sandin, P and Moula, P (2015). Modern biotechnology, agriculture and ethics. *J. Agric. Environ. Ethics, 28(5)*: 803-806.

Shukoor, M; Shuhada, N; Tamrin, M; Bahri, S; Guan, N Y; Nata, M S and Hazwani, D (2018). Development of new hard hat dimensions using user-centered design approach among oil palm harvesters. *Work, 60(1)*: 129-134.

Singh, R; Low, E T L; Ooi, L C L; Ong-Abdullah, M; Ting, N C; Nagappan, J; Nookiah, R; Amiruddin, M D; Rosli, R; Manaf, M A A and Chan, K L (2013a). The oil palm SHELL gene controls oil yield and encodes a homologue of SEEDSTICK. *Nature, 500(7462)*: 340-344.

Singh, R; Ong-Abdullah, M; Low, E T L; Manaf, M A A; Rosli, R; Nookiah, R; Ooi, L C L; Ooi, S E; Chan, K L; Halim, M A and Azizi, N (2013b). Oil palm genome sequence reveals divergence of interfertile species in old and new worlds. *Nature, 500(7462)*: 335-339.

Singh, R; Low, E T L; Ooi, L C L; Ong-Abdullah, M; Ting, N C; Nookiah, R; Ithnin, M; Marjuni, M; Mustaffa, S; Yaakub, Z; Amiruddin, M D; Manaf, M A A; Chan, K L; Halim, M A A; Sanusi, N S N M; Lakey, N; Sachdeva, M; Bacher, B; Garner, P A; MacDonald, J D; Smith, S W; Wischmeyer, C; Budiman, M A; Beil, M; Stroff, C; Reed, J; Van Brunt, A; Berg, H; Ordway, J M and Sambanthamurthi, R (2020). Variation for heterodimerization and nuclear localization among known and novel oil palm SHELL alleles. *New Phytol., 226(2)*: 426-440.

Slack, N; Chambers, S and Johnston, R (2010). *Operations Management*. Pearson Education. p. 270.

Sniezko, R A and Koch, J (2017). Breeding trees resistant to insects and diseases: Putting theory into application. *Biol. Invasions, 19(11)*: 3377-3400.

Stevanato, P; Trebbi, D; Panella, L; Richardson, K; Broccanello, C; Pakish, L; Fenwick, A L and Saccomani, M (2015). Identification and validation of a SNP marker linked to the gene HsBvm-1 for nematode resistance in sugar beet. *Plant Mole. Biol. Rep., 33(3)*: 474-479.

Sutton, K (1989). Malaysia's FELDA land settlement model in time and space. *Geoforum, 20(3)*: 339-354.

Takata, M (2008). The Development and Current Issues of the Malaysian Palm Oil Industry Dissertation 74, 29. Department of Social and Environmental Studies. Fukuoka Institute of Technology, Japan.

Tomlinson, I (2013). Doubling food production to feed the 9 billion: A critical perspective on a key discourse of food security in the United Kingdom. *J. Rural Studies, 29*: 81-90.

Tzachor, A (2019). The future of feed: Integrating technologies to decouple feed production from environmental impacts. *Ind. Biotechnol., 15(2)*: 52-62.

Urnov, F D; Ronald, P C and Carroll, D (2018). A call for science-based review of the European court's decision on gene-edited crops. *Nat. Biotechnol., 36(9)*: 800.

Vanderzande, S; Piaskowski, J L; Luo, F; Edge-Garza, D; Klipfel, J; Schaller, A; Martin, S and Peace, C (2018). Crossing the finish line: How to develop diagnostic DNA tests as breeding tools after QTL discovery. *J. Hortic, 5(228)*: 2376-0354.

Van Gelder, J W (2004). *Greasy Palms: European Buyers of Indonesian Palm Oil*. Friends of the Earth, London. 64 pp.

Van Nocker, S and Gardiner, S E (2014). Breeding better cultivars, faster: Applications of new technologies for the rapid deployment of superior horticultural tree crops. *Horticulture Research, 1(1)*: 1-8.

Wahid, M B and Simeh, M A (2009). Issues related to production cost of palm oil in Malaysia. *Oil Palm Industry Economic J., 9(2)*: 1-12.

Wannemuehler, S D; Luby, J J; Yue, C; Bedford, D S; Gallardo, R K and McCracken, V A (2019). A cost-benefit analysis of DNA informed apple breeding. *HortScience, 54(11)*: 1998-2004.

Wannemuehler, S D; Yue, C; Hoashi-Erhardt, W K; Gallardo, R K and McCracken, V (2020). Cost-effectiveness analysis of a strawberry breeding program incorporating DNA-informed technology. *HortTechnology, 30(3)*: 365-371.

Zimmer, Y (2016). Competitiveness of rapeseed, soybeans and palm oil. *J. Oilseed Brassica, 1(2)*: 84-90.

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ALL-IN-ONE COMPREHENSIVE EXTRACTION OF METABOLITES, PROTEINS AND RIBONUCLEIC ACID FOR THE RAPID ANALYSIS OF OIL PALM SYSTEMS BIOLOGY

SYAHANIM SHAHWAN[1]*; ABRIZAH OTHMAN[1]; ZAIN NURAZAH[1]; NURUL LIYANA ROZALI[1] and UMI SALAMAH RAMLI[1]

ABSTRACT

*Oil palm (*Elaeis guineensis *Jacq.) systems biology offers a comprehensive view of the plant system by employing a holistic multi-omics approach encompassing the molecular data at various hierarchical levels. Sample limitation and the importance of integrating all molecular data with minimal variation, led to the development of sequential extraction of biomolecules fractions from a single undivided biological sample. Oil palm root was subjected to solvent extraction with methanol-chloroform-water to recover metabolites of diverse polarity. The resultant pellet was subjected to buffer and solvent partitioning to obtain RNA and proteins. RNA extracted from the oil palm root showed a recovery of 180.25 ng mg^{-1}, with an A260:A280 ratio ranging between 1.9-2.0 and RNA integrity number (RIN) value of 6.7. Co-extracted proteins resulted in a recovery of 29.28 µg per 100 mg fresh weight (FW) tissue and revealed a total of 1852 identified proteins in the oil palm root. Polar metabolites revealed approximately 40 metabolite peaks, and non-polar metabolites with two major fatty acid groups* i.e.*, saturated and unsaturated fatty acids at 55.4% and 38.6%, respectively. This protocol demonstrated an advancement of extraction protocols for oil palm root biomolecules, which will consecutively expedite the establishment of various multi-omics platforms.*

Keywords: all-in-one extraction, fatty acids, oil palm biomolecules, proteins, RNA.

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INTRODUCTION

A comprehensive study and analysis of complex biological components within tissues or cells, provides a holistic approach to comprehend complex cellular processes and functions (Bruggeman and Westerhoff, 2007). Biological input obtained from the various omics platforms, including transcriptomics, proteomics and metabolomics are integrated to understand the interrelationship between networks of biological processes (Potters, 2010). A new improved breeding material is always in demand by oil palm stakeholders. Thus, improving oil palm varieties via its genetics information is crucial and need to be materialised by advanced biotechnology (Kushairi *et al.*, 2019). Omics-based studies have been fully endeavoured in oil palm research with extensive discoveries of transcripts (Avila-Mendez *et al.*, 2019; Bahari *et al.*, 2018; Low *et al.*, 2008; Ooi *et al.*, 2015; Rosli *et al.*, 2018; Singh *et al.*, 2013; 2014; Tee *et al.*, 2013; Xia *et al.*, 2014), proteins (Hassan *et al.*, 2019; Jeffery Daim *et al.*, 2015; Lau *et al.*, 2018; Ooi *et al.*, 2015; Syahanim *et al.*, 2013) and metabolites (Rozali *et al.*, 2017; Tahir *et al.*, 2012; Zain *et al.*, 2013). It is imperative to understand the molecular mechanisms governing the complex diversity of biomolecules and dynamics in oil palm traits such as yield, height, quality and disease resistance using

[1] Malaysian Palm Oil Board,
6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.

* *Corresponding author e-mail: syahanim@mpob.gov.my*

different oil palm tissues including leaf, root, fruit, seed, trunk and basal stem, which can be deployed in breeding programmes. Transcriptomic analysis has been widely used in exploring the expression of mRNA in oil palm clonal materials under various biotic and abiotic stress conditions. As reported by Avila-Mendez *et al.* (2019), transcriptional analysis has provided an overview of the genes involved in the molecular response of the oil palm clones to *Phytophra palmivora*. Shearman *et al.* (2019) analysed expression data for all transcripts in flowers and fruits of mantled and normal oil palm clones in order to identify differentially expressed genes between these two populations.

Integration of knowledge driven by omics-based datasets has been initiated in order to understand biological system/responses under favourable or unfavourable conditions. Two or more omics datasets have been utilised to dissect strong and novel biomolecules candidates associated with the molecular process of interest. Pairwise analysis of metabolite and transcript datasets of potato tuber systems revealed significant correlation and included several strong correlations to novel nutritionally important metabolites (Urbanczyk-Wochniak *et al*., 2003). Gene-to-gene interaction and metabolite-to-gene networking was elucidated in *Arabidopsis thaliana* grown under sulphur deficiency, via integrated metabolomics and transcriptomics (Hirai *et al.*, 2005). Omics-based datasets have also been used in medicinal plant research and revealed novel biomolecules which were further functionally characterised (Chen and Facchini, 2014; Gesell *et al*., 2009; Hagel and Facchini, 2013; Liscombe *et al*., 2009; Marques *et al*., 2014; Miettinen *et al*., 2014; Pienkny *et al*., 2009; Rai *et al*., 2017; Rischer *et al*., 2006; Rohani *et al*., 2016; Saito, 2013; Srivastava *et al*., 2013; Udomsom *et al*., 2016; Ziegler *et al*., 2005; 2006). Integration of omics-based datasets was also used for dissecting biological response of *Brassicaceae* towards UV-B irradiation (Tohge *et al*., 2016). Multi-omics datasets of DNA methylome, transcriptome and metabolome were integrated and provided novel insight into the regulation of cotton fibre development by epigenetic mechanisms (Wang *et al*., 2016). Metabolite-based genome-wide-association-study (mGWAS), has been used to dissect the genetic control of plant metabolism in plants (Luo, 2015; Matsuda *et al*., 2015).

Analysis of biomolecules to understand the biochemical reactions within a cell involving transcripts, proteins and metabolites in oil palm systems biology is limited. Vidal (2009) indicated that the interaction of macromolecules and metabolites in cells or organs of an organism form a multi-scale dynamic complex system that is fundamental to biological processes. An integrated approach was developed in order to comprehend oil palm biomolecules as well as the compendium function of biological elements that are part of oil palm systems biology. At present, most of the integrative experiments involve the split-sample-study design where biological samples are divided into multiple parts with each part generating an omics dataset (Rai *et al*., 2017). Integration of the data is done later by using dedicated bioinformatics software tools to ascertain the desired interactions and functions in systems biology. However, this approach has limitations as it may introduce bias that leads to misinterpretation and miscorrelation as different populations of RNA, proteins and metabolites that might be expressed differently in the different sets of tissues (Eckert *et al*., 2012; Li *et al*., 2010; McConnell and Barton, 1998). Rai *et al.* (2017) iterated that combining multi-omics datasets poses great obstacles due to the requirements/criteria for scaling, noise removal, sensitivity and resolution for each data type variable. The authors also explained that different omics outputs result in lists of unrelated entities, and appropriate statistical tools need to be carefully chosen, in establishing biologically relevant relationships between cellular components. Experimental design is also a pivotal component, which significantly impacts the quality of omics-based datasets (Cavill *et al*., 2016). Thus, an integrated extraction protocol was designed in this study to extract RNA, proteins and metabolites in parallel. This protocol was also applied to overcome the major drawback of inadequate sample volume to perform various experiments via omics platforms. Chomczynski (1993) described the simultaneous extraction of RNA, DNA and proteins from human mammary epithelial cells, rat mammary tissues and mouse liver. Later, similar protocols were also employed to extract biomolecules from leaves and roots of plant tissues. For instance, a novel method for successful integrated extraction of metabolites, proteins and RNA from *Arabidopsis thaliana* was reported by Weckwerth *et al.* (2004). A universal protocol for the multiple molecular extraction of biomolecules (metabolites, DNA, total RNA, large RNA, small RNA, and proteins) from a single sample of a wide range of species such as *Populas*, *Pines*, *Arabidopsis* and *Chlamydomonas* was also described, allowing for a broad range of transcriptional studies, metabolite profiling and shotgun proteomics (Valledor *et al*., 2014). In addition, simultaneous extraction of biomolecules was employed in cell lines for a comprehensive molecular analysis of biological systems (Sapcariu *et al*., 2014; Vorreiter *et al*., 2016). A sequential isolation of metabolites, RNA, DNA and proteins applicable to microbial ecology was also presented to facilitate systematic multi-omics analysis and enable meaningful data integration (Roume *et al*., 2013).

This article describes an all-in-one extraction protocol of metabolites, proteins and RNA from a single oil palm root sample. The developed protocol

could then be applied to understanding the oil palm systems biology through the integration of multi-omics data: Transcriptomics, proteomics and metabolomics simultaneously, thus, minimising the variance that would result from multiple experiments.

MATERIALS AND METHODS

Plant Materials

One-year old oil palm (Deli *dura* x Avros *pisifera*) root samples were obtained from MPOB Kluang Research Station, Johor, Malaysia in three biological replicates. The roots were cleared of soil and washed briefly. Another washing step was performed using sterile water, and primary roots were cut into small pieces and then snap frozen using liquid nitrogen and stored at -80°C until use.

All-in-one-extraction: At a Glance

Figure 1 represents a schematic overview of the experimental workflow of the protocol which can be easily adjusted in accordance to the required quantity of samples. At a glance, solvent extraction using a mixture of methanol, chloroform and water (2.5:1.0:0.5, v/v/v) was used to extract metabolites of various range of polarities. The resulting pellet was washed and subjected to protein extraction buffer/100% phenol/20% (v/v) chloroform-based extraction for proteins and RNA isolation. Later, buffer and phenol phases were precipitated to obtain the oil palm root RNA and protein, respectively. Qualitative analysis of the extracted biomolecules was evaluated using common and established analysis.

All-in-one-extraction: Metabolites

The integrated extraction protocol was developed for oil palm based on Valledor *et al.* (2014) and Weckwerth *et al.* (2004) with minor modification. Oil palm root (100 mg) was ground into a homogenous fine powder using mortar and pestle in liquid nitrogen. After adding a fixed volume (2.0 mL) of pre-cooled (-20°C) solvent mixture of methanol, chloroform and water (2.5: 1: 0.5, v/v/v), the mixture was thoroughly vortexed for 30 min and then centrifuged at 15 000 g for 5 min. The resulting pellet was washed with 2.0 mL of pre-cooled chloroform/methanol mix (1:1, v/v). The solvent extracts were combined and used for metabolite analysis accordingly. Polar and non-polar components were separated by adding 5.0 mL of distilled water.

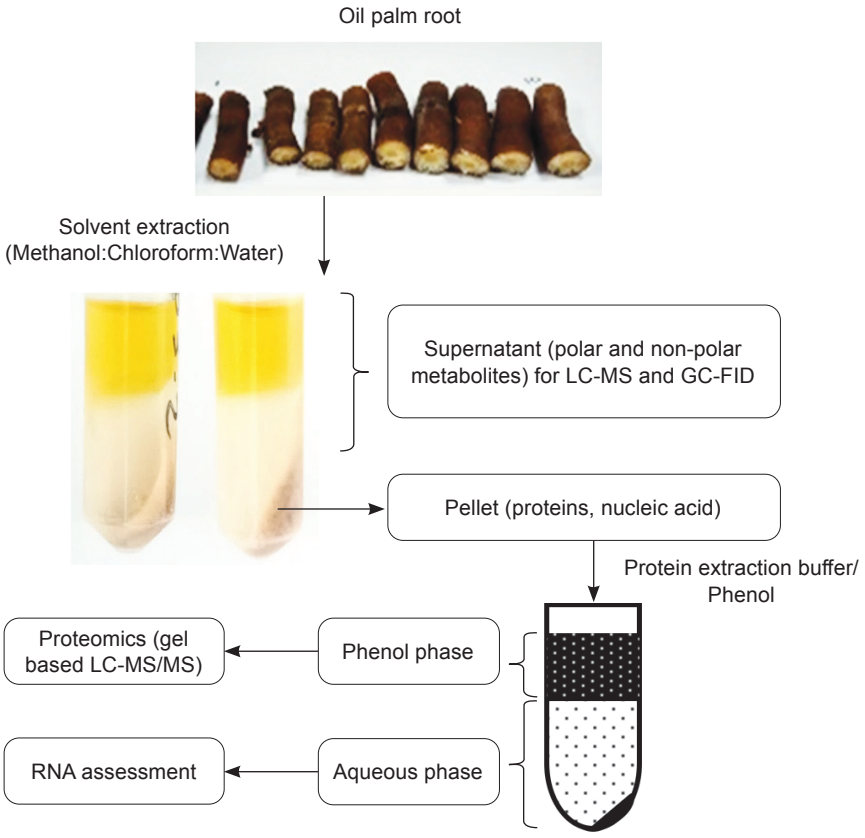

Figure 1. Schematic presentation of all-in-one extraction protocol of metabolites, proteins and RNA from the oil palm root.

All-in-one-extraction: RNA and Proteins

After removing the solvent phase from the tube, the pellet which consisted of nucleic acid (DNA/RNA), proteins, starch, membrane and cell wall components was resuspended with 1.0 mL protein extraction buffer. We tested three different buffers based on previous reports in order to identify the most suitable protein extraction buffer which gave reproducible results and minimised protein losses. The methods employed were adopted from Valledor *et al*. (2014); Weckwerth *et al*. (2004) and the modified method of Syahanim *et al*. (2013), and the buffers were designated as buffer A, B and C, respectively. The protein extraction buffer, i) buffer A consisted of 0.10 M Tris-hydrochloride (Tris-HCl) pH 8.8, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.9 M sucrose and 0.4% (w/v) mercaptoethanol, ii) buffer B consisted of 0.10 M Tris, 25 mM EDTA, 0.9 M sucrose and 0.4% (w/v) dithiothreitol (DTT) and iii) buffer C consisted of 0.05 M Tris, 0.5 M sodium chloride (NaCl), 0.5% (v/v) sodium dodecyl sulphate (SDS), 0.9 M sucrose and 0.4% (w/v) mercaptoethanol. Buffer C was added with 0.5 M NaCl to improve the protein solubility and to mimic the physiological condition. A tablet of protease inhibitor cocktail (Roche) was added to 10 mL protein extraction buffer mix to inhibit protease activity. The pellet-buffer mix was sonicated for 10 min at 4°C and added with 2.0 mL of Tris-buffered phenol. Then, the buffer mix was vortexed for 10 min and centrifuged at 16 000 g for 10 min, portioned to two phases *i.e.*, phenol phase and aqueous phase.

For ribonucleic acid extraction, 0.2 mL of chloroform was added to the aqueous phase. The mixtures were centrifuged at 16 000 g for 10 min at 4°C. Another 0.2 mL was added to the RNA-upper buffer phase and mixed briefly. After centrifugation at 16 000 g for 5 min, a total of 0.04 mL of acetic acid and 1.0 mL of ethanol were added to the aqueous phase for RNA precipitation overnight at -20°C. After centrifugation, the pellet was washed with 0.2 mL of 3 M sodium acetate and two times with 0.2 mL of 70% ethanol. The remaining pellet was re-dissolved in 50 µL RNase-free water.

The phenol phase which contained proteins was separated from the RNA-aqueous phase followed by back-extraction with an equal volume of protein extraction buffer. The phenol phase was precipitated with 5 volumes of pre-cooled 0.1 M ammonium acetate in 100% (v/v) methanol. The mixture was kept at -20°C for 16 hr and centrifuged at 12 000 g for 30 min at 4°C. The pellet was rinsed twice in 0.2 mL of methanol and twice with pre-cooled acetone. The protein pellet was dried for 5 min and dissolved in 0.5-1.0 mL solubilisation buffer containing 7 M urea and 0.02 M DTT. Protein quantification was performed using Qubit Protein Assay Kit (Thermo Fisher Scientific, Wilmington, DE, USA) with bovine serum albumin (BSA) as standard at concentration range from 0 mg mL^{-1} to 5 mg mL^{-1}.

Polar Metabolite Analysis by Liquid Chromatography-Mass Spectrometry (LC-MS)

The polar phase containing polar metabolites was subjected to liquid chromatography-mass spectrometry (LC-MS) analysis. One microliter of the polar phase was injected to an Ultimate 3000 HPLC System coupled to MicrOTOF-Q™, a quadrupole/time of flight (Q/TOF) mass spectrometer (Bruker Daltonics, GmbH, Bremen, Germany). Separation was obtained on a Reverse-Phase Acclaim Polar Advantage II column (C18, 4.6 x 250 mm length, 5 µm particle size) (Thermo Fisher Scientific, Wilmington, DE, USA) at 37°C with a gradient elution programme of 1 mL min^{-1} flow rate with an increasing ACN/acetic acid gradient. All samples and replicates were continuously injected as one batch in random order to discriminate between technical and biological variations. Additionally, pooled samples were used as quality control (QC) and injected at regular intervals throughout the analytical run. The Q/TOF MS analysis was performed in negative electrospray ionisation and controlled by the HyStar Application version 3.2 software (Bruker Daltonics, GmbH, Bremen, Germany). The column effluent was set at 1.0 mL min^{-1}. A split ratio of 1:4 was used generating a final flow rate of 250 µL min^{-1}. Nitrogen was used as nebulising gas at 4.1 bar and 9.0 l min^{-1} flow rate. The temperature and voltage of capillary were set at 200°C and +3.5 kV, respectively. The full MS scan covered the mass range of *m*/*z* 50 to 1000.

The LC-MS data were processed through the Data Analysis 4.2 software (Bruker Daltonics, GmbH, Bremen, Germany). Find Molecular Features was applied to the raw data under these parameters: Signal to noise ratio (S/N) threshold was set to 5.0, correlation coefficient was set to 0.7, minimum compound length was set to 10 spectra and smoothing width was set to 1.0. The data was evaluated in a time range from 0 to 40 min and in a mass range from *m*/*z* 50 to 1000.

Non-polar Metabolite Analysis by Gas Chromatography-Flame Ionisation Detector (GC-FID)

The non-polar phase containing non-polar metabolites which included fatty acids was subjected to GC-FID (Clarus 500, Perkin Elmer). The non-polar phase was dried for fatty acid methyl esterification based on Yuan (2016). A total of 2 mL of 2.5% (v/v) sulphuric acid in methanol and 0.5 mL of toluene were dispensed to the extract prior to heating at 80ºC for an hour. Then, 2 mL of 0.9%

(w/v) NaCl and 1 mL of hexane were added and mixed together. The upper phase was transferred into a new vial and dried under nitrogen blower. Dried fatty acid methyl ester (FAME) of oil palm root tissue was dissolved in 100 μL of hexane. One microliter of the derivatised fatty acid was then analysed by GC-FID equipped with a fused-silica capillary column (BPX 70: 30 m length, 0.32 mm diameter and 0.25 μm film thicknesses) (SGE Analytical Science). The injector temperature was fixed at 250ºC and the oven temperature program was held at 80ºC for 1 min and then ramped at 10°C min^{-1} to 235ºC for 1 min.

The individual FAME peaks were identified by comparing the retention time of the FAME peaks with the reference standards. The peak area of the chromatogram was exploited to calculate the percentage of the total fatty acid in the oil palm root.

RNA Integrity Assessment

Total RNA was prepared according to the Agilent RNA 6000 Nano quick start guide and analysed using the Agilent BioAnalyser 2100 (Agilent Technologies Inc). The RNA integrity number (RIN) was generated by the 2100 Software version B.02.08.SI648.

Protein Analysis by Gel-based Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Gel-based LC-MS was performed on protein samples using 12% polyacrylamide gel via Bio-Rad Mini-Protean III equipment (Bio-Rad) at 50 V for 15 min followed by 200 V until 1 cm mark below the stacking gel as described by Valledor and Weckwerth (2014). Protein gels were stained for 30 min with Coomassie Brilliant Blue and destained four times for 80 min with destaining solution. Each lane was cut into two fragments and chopped into small pieces of 1 mm size. Blue colour was removed by soaking the gel pieces in 1 mL of 25 mM ammonium bicarbonate (NH_4HCO_3) for 15 min at 37°C and replaced with 1 mL of 25 mM NH_4HCO_3 in 50% (v/v) acetonitrile (ACN). The gel pieces were incubated at 37°C for 15 min. Solvent was discarded and the gel pieces were dehydrated with 200 μL of ACN for 5 min at room temperature. The gel pieces were dried using a SpeedVac followed by tryptic digestion at 12.5 ng μL^{-1} in trypsin buffer consisted of 25 mM NH_4HCO_3, 10% (v/v) ACN and 5 mM calcium chloride ($CaCl_2$). The peptide was digested at 37°C for 16 hr. The peptides were further extracted in three consecutive steps using 50% (v/v) ACN/1% (v/v) formic acid for two times and 90% (v/v) ACN/1% (v/v) formic acid. Supernatant was pooled and dried in the SpeedVac for 5 min. Peptide desalting was performed using a C18-ZipTip (Millipore, Bedford, MA) according to manufacturer's recommendation.

The peptide was analysed using EASY-nLC 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) coupled with Orbitrap Fusion mass spectrometry (Thermo Fisher Scientific, Wilmington, DE, USA). Chromatographic separations were performed on a reversed-phase analytical column (EASY-Spray Column Acclaim PepMap™ C18 100 A^0, 2 μm particle size) (Thermo Fisher Scientific, Wilmington, DE, USA). Peptide separation was achieved via gradient settings at 5% (v/v) to 40% (v/v) solvent B (0.1% (v/v) formic acid in ACN) for 91 min. The peptides were loaded onto the column with 95% (v/v) solvent A (0.1% (v/v) formic acid in LC-MS grade water) and desalting was performed for 1 min with 95% (v/v) solvent A using reverse phase C18 trapping column. Tandem mass spectra were generated using the Orbitrap Fusion mass spectrometry (Thermo Fisher Scientific, Wilmington, DE, USA) and protein identification was performed using the Thermo Fisher Scientific™ Proteome Discoverer™ Software Version 2.1 against the oil palm in-house database and oil palm dataset available at UniProt. Database search parameter included the following: up to two missed cleavage sites was allowed; variable modification of oxidation (M), deamidation of asparagine (N) and glutamine (Q) and fixed modification, carbamidomethylation (C); peptide mass tolerance was 10 ppm. All peptides were validated using the percolator® algorithm, based on q-value less than 1% false discovery rate (FDR).

RESULTS

Obtaining adequate biomolecules samples which are reproducible and of high quality, and minimising variables are always top priority in any biological research. Oil palm is the most productive oil-bearing crop and research to understand the systems biology, especially relating to its fruit development and oil productivity, is of pertinent interest. Generally, oil palm RNA, DNA, proteins and metabolites are extracted independently based on research goals. Oil palm, as other free-standing plants, is supported by its root system, which acts as the main source of nutrient for growth and development. In this study, oil palm root samples were used in the development of an all-in-one extraction protocol. Later, this protocol could be applied to other part of palm tissues such as mesocarp and leaf. The all-in-one extraction protocol using oil palm root samples was adopted from Syahanim *et al.* (2013); Valledor *et al.* (2014) and Weckwerth *et al.* (2004) with minor modification by adding 0.5 M sodium

chloride to the protein extraction buffer mix and additional chloroform wash during RNA purification step. The protocol allowed for fast and reproducible extraction of metabolites, proteins and RNA from the oil palm root samples.

Metabolite Analysis of Oil Palm Root Samples

High throughput LC-MS technique aims at analysis and identification of small molecules involved in metabolic reactions. Here, polar phase derived from the oil palm root was subjected to LC-MS as a benchmark of wide coverage identification of metabolites. *Figure 2* shows base peak chromatogram of oil palm root polar extract generated from LC-MS analysis. The chromatogram shows good separation and coverage of metabolites. This approach has also been established for the detection of metabolic signatures in biological samples (Zhou *et al.*, 2012). Further analysis was also conducted to the non-polar phase by measuring the fatty acid content in the oil palm root using GC-FID. Heptadecanoic acid (C17:0) was used as an internal standard. Oil palm root consists of two major fatty acids *i.e.*, polyunsaturated and unsaturated fatty acids at 55.40% and 38.60%, respectively. The fatty acid analysis showed six major fatty acids (*Figure 3*), which were palmitic acid (1.75% ± 0.04), stearic acid (55.36% ± 0.04), oleic acid (4.00% ± 0.05), linoleic acid (8.64% ± 0.08), linolenic acid (23.14% ± 0.31) and eicosanoic acid (1.08% ± 0.02) with coefficient variation (CV) percentage less than 3.00%, respectively. The low percentage of CV showed the mean value for the peak area for each biological replicate samples were quite close and reproducible.

RNA Quality Assessment of Oil Palm Root

RNA was extracted twice from the buffer phase by using chloroform. The quality of RNA derived from oil palm root was assessed using a NanoDrop One system (Thermo Scientific, Wilmington, DE, USA) and the purity was estimated by the A260:A280 ratio. The RNA purity derived from buffer A, B and C was 1.1, 1.4 and 1.9, in which consisted a total of 12.66 ± 0.84 ng mg^{-1} FW^{-1}, 32.93 ± 7.67 ng mg^{-1} FW^{-1} and 180.25 ± 6.35 ng mg^{-1} FW^{-1} of RNA, respectively. Total RNA from oil palm root extracted using Buffer C gave the highest RNA yield and acceptable purity. Thus, oil palm root protein and RNA extracted from buffer C were further analysed for protein identification and RNA integrity determination. A good RIN value is required for downstream applications such as sequencing and real time PCR. RIN values range from 1.0 (degraded) to 9.0 (intact). RIN value is calculated and generated from Agilent 2100 Bioanalyser. The integrity of RNA extracted from the oil palm root using sequential protocol generated a RIN value of 6.7 (*Figure 4*). Therefore, our results suggest that the all-in-one extraction method employed in this study is capable of producing good quality of RNA from oil palm root tissue adequate for downstream analysis.

Oil Palm Root Protein Assessment

The all-in-one protocol employed protein extraction buffers and phenol-based extraction to extract proteins from plant tissues as a starting material (Valledor *et al.*, 2014; Weckwerth *et al.*, 2004). In addition, Syahanim *et al.* (2013) has extracted

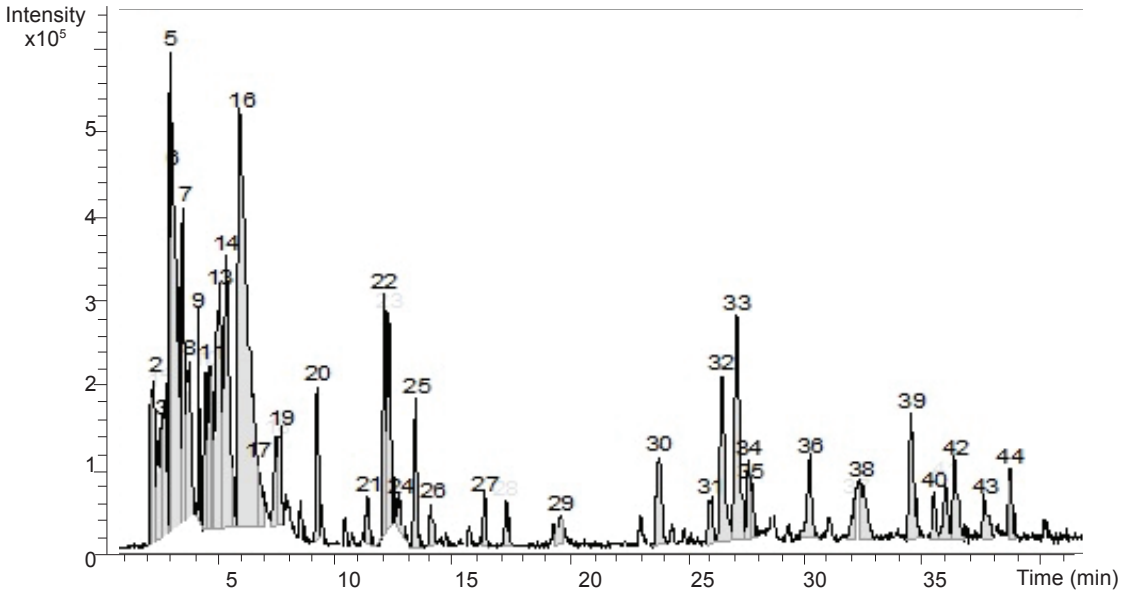

Figure 2. Liquid chromatography mass spectrometry (LC-MS) base peak chromatogram (BPC) of oil palm root extract in negative ionisation mode. More than 40 peaks were detected in the oil palm root sample.

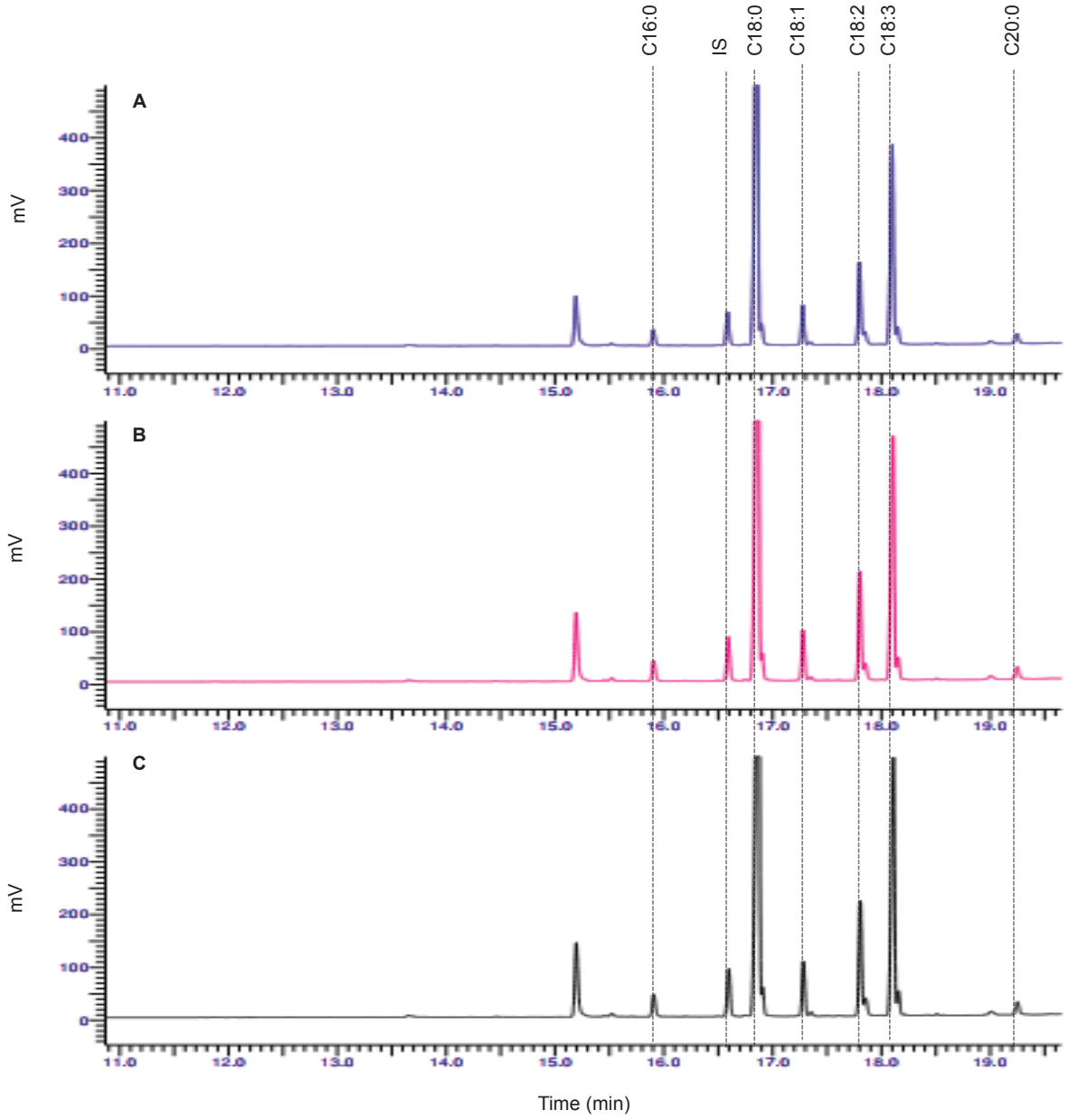

Figure 3. GC-FID chromatogram of fatty acid methyl esters (FAME) from the oil palm root in triplicate (Root A, Root B and Root C). Oil palm root consists of C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), C18:3 (linolenic acid) and C20:0 (eicosanoic acid) fatty acids. C17:0 (heptadecanoic acid) was used as an internal standard (IS).

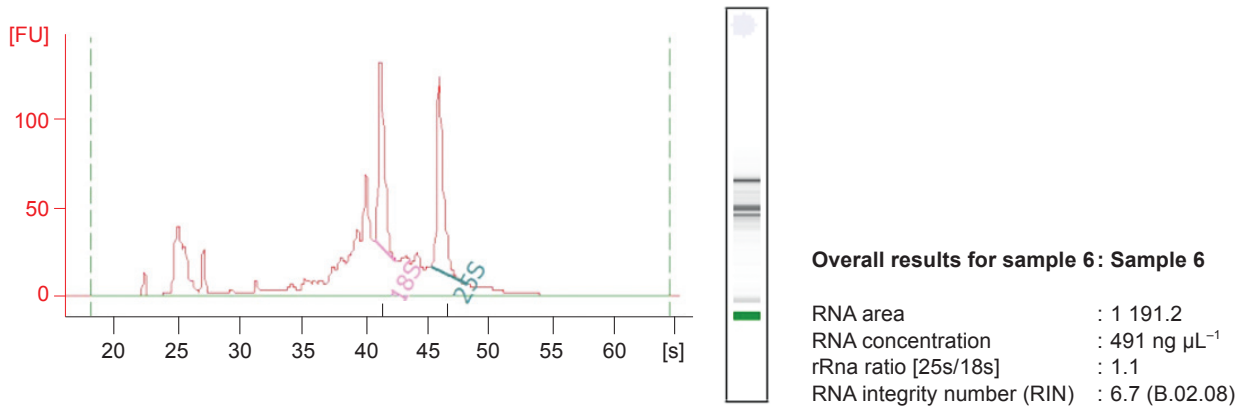

Figure 4. A representative image of electropherogram of total RNA sample from the oil palm root evaluated using the Bioanalyser (Agilent Technologies Inc).

proteins from oil palm roots using phenol-based extraction method. Thus, the protein extraction buffer formulation was used based on these three papers and designated as protein extraction buffer A, B and C, respectively.

Accordingly, the protein extracts obtained from protein extraction buffer A, B and C were quantified using Qubit Protein Assay Kit. Referring to BSA (5 mg mL^{-1}) as reference protein, a standard curve was plotted to calculate the protein concentration. Protein recoveries of 17.32 ± 1.21 µg (buffer A), 29.28 ± 2.32 µg (buffer B) and 27.40 ± 2.19 µg (buffer C) per 100 mg oil palm root were recorded, respectively (*Table 1*). Protein extraction buffer based on Weckwerth *et al.* (2004) (buffer B) demonstrated highest protein recovery with a CV of 7.92% followed by protein extraction buffer based on Syahanim *et al.* (2013) with slight modification with a CV of 7.99%. Analysis of one-way (ANOVA) for the root protein recovery showed a significant difference (p<0.005) between protein extracted with buffer A, B and C. The quality of extracted proteins was evaluated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie Brilliant Blue staining (Vorreiter *et al.*, 2016). *Figure 5* shows the oil palm root protein separation in one dimensional SDS-PAGE (12%). Visual comparison of protein bands in SDS-PAGE lanes corresponding to oil palm root protein extracts derived from protein extraction buffer A, B and C demonstrated distinct similarities. Gel-liquid chromatography fractionation coupled to mass spectrometry was performed on proteins extracted from buffer C in three biological replicates. Tandem mass spectrometry demonstrated a total of 90 182 MS spectrum was generated from the tryptic peptide of the oil palm root. A peptide spectrum matching was conducted against oil palm database collections derived from in house datasets and public repository and revealed a total 1852 ± 5 identified proteins in triplicate. All peptides were validated using the percolator® algorithm, based on q-value less than 1% FDR. Multi-scatter plot with Pearson correlation coefficient were above 0.95, demonstrating a positive correlation in between three biological replicates (*Figure 6*). Protein classification was performed via Blast2Go (Go version: 1 January 2020) and the identified oil palm root protein datasets were classified in three groups of biological process, cellular component and molecular function (*Figure 7*). Digested proteins derived from simultaneous extraction were

TABLE 1. OIL PALM ROOT PROTEIN RECOVERY EXTRACTED USING THREE DIFFERENT PROTEIN EXTRACTION BUFFERS

Protein extraction buffer	Protein recovery (µg mg^{-1} FW)	Coefficient variation, CV (%)	Reference
A	17.32 ± 1.21*	6.98	Valledor *et al.* (2014)
B	29.28 ± 2.32*	7.92	Weckwerth *et al.* (2004)
C	27.40 ± 2.19*	7.99	Syahanim *et al.* (2013) with slight modification

Note: Root sample: 100 mg.
*Average and standard deviation were calculated from triplicate samples.

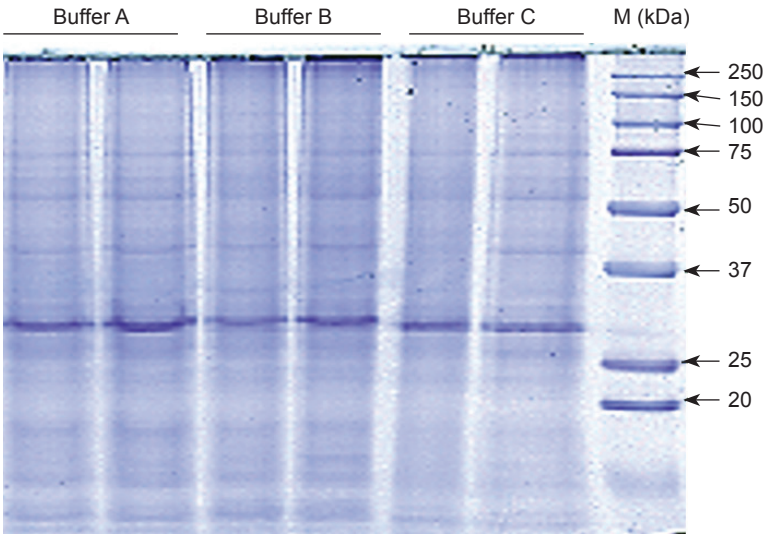

Figure 5. One dimensional gel electrophoresis of the oil palm root samples (25 µg) extracted using protein extraction buffer A, B and C, respectively. M: Kaleidoscope protein marker.

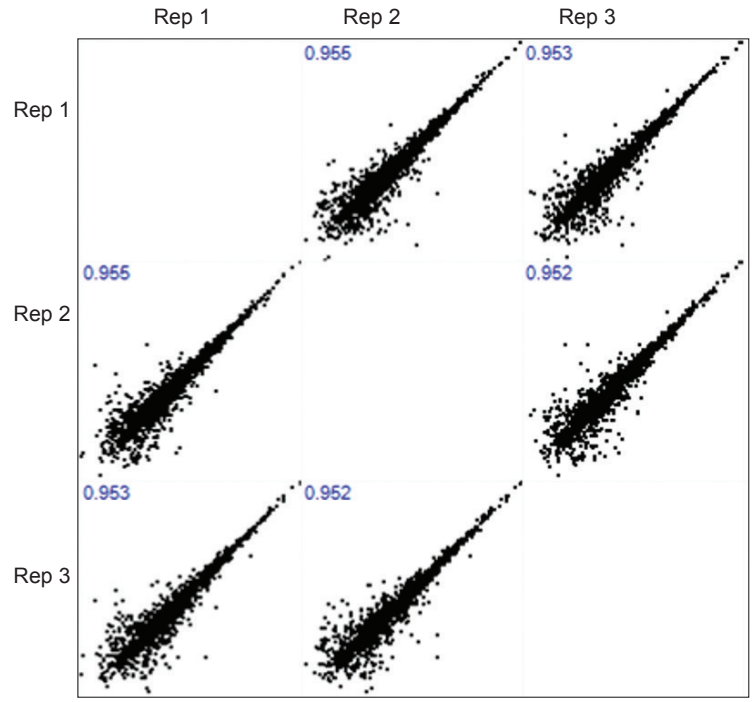

Figure 6. Multi-scatter plots with Pearson correlation values of above 0.95, suggesting a good correlation between data obtained from biological replicate samples of oil palm root.

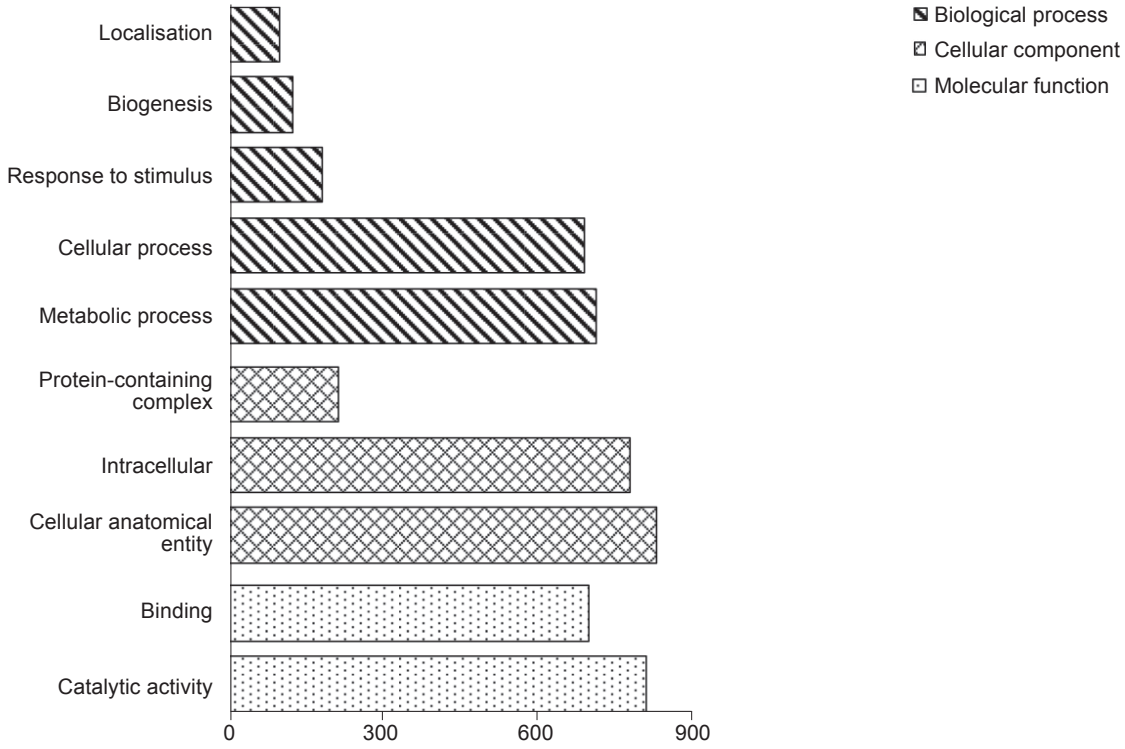

Figure 7. Annotation based on Gene Ontology terms using Blast2Go of amino acid sequences derived from the oil palm root proteome.

subjected to high resolution accurate mass (HRAM) mass spectrometry as demonstrated by Vorreiter *et al.* (2016) and Weckwerth *et al.* (2004) and revealed 1091 and 297 identified proteins from the human T-cell lines and *Arabidopsis* sample, respectively.

DISCUSSION

Several reports have demonstrated the compatibility of biomolecule components extracted using simultaneous extraction protocols for various multi-

omics approaches. Chomczynski (1993) invented a mono-phase solution which consisted of acid guanidine thiocyanate-phenol-chloroform for the single-step simultaneous isolation of RNA, DNA and proteins from animal tissues. Electrophoretic pattern of RNA showed two distinct ribosomal bands, as an indicator for authenticity of the total RNA. A column-based commercial kit was also tested for co-extraction of RNA and proteins as described by Morse *et al.* (2006). The team used RNeasy® Mini Kit (QIAGEN) to extract RNA from cultured cell lines. After column centrifugation, the flow-through was retained and subjected to protein precipitation. By performing this procedure, concurrent extraction of RNA and proteins from the experimental sample was efficiently conducted. Method optimisation for simultaneous extraction of RNA, DNA, proteins and metabolites was reported using human T-cells and hepatocytes cell lines in which three methods were tested (Vorreiter *et al.*, 2016). The first method was based on Weckwerth *et al.* (2004). For comparison, a second method was tested using commercial buffer, TRI REAGENT™ (www.sigmaaldrich.com) according to the manufacturer's protocol. The third method employed phenol/chloroform for RNA, DNA and proteins with an additional step included to extract metabolites using methanol/chloroform. The suitability of the extraction methods for the biomolecules was evaluated and it was observed that the third method adequately produced good quality of DNA, RNA, proteins and metabolites from the cell lines. Morgenthal *et al.* (2005) and Weckwerth *et al.* (2004) have demonstrated a novel extraction protocol for integrated extraction of biomolecules from *Arabidopsis thaliana* leaves. Valledor *et al.* (2014) used several plants to develop an improved universal protocol for isolation of biomolecule components in a single sample of *Chlamydomonas*, *Arabidopsis*, *Populus* and *Sinus*. Additionally, metabolite extraction was conducted based on Weckwerth *et al.* (2004) while subsequent isolation of proteins and RNA was performed using commercial kits.

Despite recent advancement in oil palm proteomics and metabolomics research (Ramli *et al.*, 2016), multi-omics analysis of the same sample of oil palm is still challenging. In this report, we describe a novel protocol for the sequential extraction of metabolites, proteins and RNA from the same undivided oil palm root sample to facilitate meaningful data integration. Simultaneous extraction of metabolites, proteins and RNA from the oil palm tissue was exploited using the established protocol by Valledor *et al.* (2014) and Weckwerth *et al.* (2004) with slight modification during RNA purification and additional protein extraction buffer formulation according to Syahanim *et al.* (2013). To isolate RNA from the oil palm samples, a classical phenol/chloroform protocol (Weckwerth *et al.*, 2004) is preferred than using a silica-based column (Valledor *et al.*, 2014) due to the rising cost of commercially available columns. However, the classical RNA extraction is prone to contamination of phenol and salts that may affect the RNA quality for downstream application. To overcome this, we introduced a second chloroform washing step, as well as a twice washing step as described by Toni *et al.* (2016). For animal and human tissues, RIN value of 9.0 is needed for further analysis. For plants, RIN value of more than 5.0 is acceptable for further downstream analysis (Fleige and Pfaffl, 2006). This is due to the fact that plant species have diverse ribosomal RNA sizes (5S, 8S, 16S, 23S and 25S) from mitochondria, cytosol and chloroplast which contribute to the complexity in RIN value. The classical RNA extraction described in this article produced adequate RNA of acceptable quality from oil palm root tissue for downstream analysis.

In the following sections, we provide an exemplary of the multi-level extraction of proteins from oil palm root using different buffers as previously reported by other groups (Syahanim *et al.*, 2013; Valledor *et al.*, 2014; Weckwerth *et al.*, 2004). However, the performance of the protein extraction buffer for the integrated extraction protocol has never been reported for an oil palm tissue. In the present study, after removing the solvent phase which contained metabolites, the remaining solid pellet was used for protein extraction. This step employed extraction buffers and phenol to extract proteins as described previously for plant tissues (Valledor *et al.*, 2014; Weckwerth *et al.*, 2004). Syahanim *et al.* (2013) also reported an independent phenol-based extraction method to extract proteins from oil palm roots. The protein extraction buffer formulation described in the above three studies involved the usage of sucrose as a phase conversion and reducing agent. The protein extraction buffer formulation reported by Weckwerth *et al.* (2004) produced the highest protein. The buffer formulation reported by Syahanim *et al.* (2013) was also included in this work, although it produced slightly lower protein recovery, the buffer used was able to extract out RNA at the highest yield and acceptable purity. This finding concluded that the protein extraction buffer formulation according to Syahanim *et al.* (2013) is the suitable buffer to be used in all-in-one extraction protocol for oil palm root samples.

Thus, the prospect of extracting metabolites, proteins and RNA from oil palm tissues using an all-in-one extraction protocol reported in this article is very attractive for achieving fast and high-quality biomolecule extracts, in a single experiment. Thus, the developed integration protocol could be explored further in other oil

palm tissues such as mesocarp and leaf. This will enable comprehensive analysis using various omics platforms to fill knowledge gaps in oil palm systems biology.

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REFERENCES

Avila-Mendez, K; Rodrigo, A; Araque, L and Romero, H M (2019). Simultaneous transcriptome analysis of oil palm clones and *Phytophthora palmivora* reveals oil palm defense strategies. *PLoS ONE, 14*: e0222774.

Bahari, M N A; Sakeh, N M; Abdullah, S N A; Ramli, R R and Kadkhodaei, S (2018). Transciptome profiling at early infection of *Elaeis guineensis* by *Ganoderma boninense* provides novel insights on fungal transition from biotrophic to necrotrophic phase. *BMC Plant Biol., 18*: 377. DOI: 10.1186/s12870-018-1594-9.

Bruggeman, F J and Westerhoff, H V (2007). The nature of systems biology. *Trend Microbiol., 15*: 45-50.

Cavill, R; Jennen, D; Kleinjans, J and Briedé, J J (2016). Transcriptomic and metabolomic data integration. *Brief. Bioinform., 17*: 891-901.

Chen, X and Facchini, P J (2014). Short-chain dehydrogenase/reductase catalyzing the final step of noscapine biosynthesis is localized to laticifers in opium poppy. *Plant J., 77*: 173-184.

Chomczynski, P (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cells and tissue samples. *BioTechniques, 15*: 532-535.

Eckert, A J; Wegrzyn, J L; Cumbie, W P; Goldfarb, B; Huber, D A; Tolstikov, V; Fiehn, O and Neale, D B (2012). Association genetics of the loblolly pine (*Pinus taeda*, Pinaceae) metabolome. *New Phytol., 193*: 890-902.

Fleige, S and Pfaffl, M W (2006). RNA integrity and the effect on the real-time qRT-PCR performance. *Mol. Asp. Med., 27*: 126-139.

Gesell, A; Rolf, M; Ziegler, J; Chávez, M L D; Huang, F C and Kutchan, T M (2009). CYP719B1 is salutaridine synthase, the CC phenol-coupling enzyme of morphine biosynthesis in opium poppy. *J. Biol. Chem., 284*: 24432-24442.

Hagel, J M and Facchini, P J (2013). Benzylisoquinoline alkaloid metabolism: A century of discovery and a brave new world. *Plant Cell Physiol., 54*: 647-672.

Hassan, H; Amiruddin, M D; Weckwerth, W and Ramli, U S (2019). Deciphering key proteins of oil palm (*Elaeis guineensis* Jacq.) fruit mesocarp development by proteomics and chemometrics. *Electrophoresis, 40*: 254-265.

Hirai, M Y; Klein, M; Fujikawa, Y; Yano, M; Goodenowe, D B; Yamazaki, Y; Kanaya, S; Nakamura, Y; Kitayama, M and Suzuki, H (2005). Elucidation of gene-to-gene and metabolite-to-gene networks in *Arabidopsis* by integration of metabolomics and transcriptomics. *J. Biol. Chem., 280*: 25590-25595.

Jeffery Daim, L D; Ooi, T E K; Ithnin, N; Mohd Yusof, H; Kulaveerasingam, H; Abdul Majid, N and Karsani, S A (2015). Comparative proteomic analysis of oil palm leaves infected with *Ganoderma boninense* revealed changes in proteins involved in photosynthesis, carbohydrate metabolism, and immunity and defense. *Electrophoresis, 36*: 1699-1710.

Kushairi, A; Ong-Abdullah, M; Nambiappan, B; Hishamuddin, E; Izuddin, Z B M N; Ghazali, R; Subramaniam, V; Sundram, S and Parveez, G K A (2019). Oil palm economic performance in Malaysia and R&D progress in 2018. *J. Oil Palm Res., 31(2)*: 165-194.

Lau, B Y C; Othman, A and Ramli, U S (2018). Application of proteomics technologies in oil palm research. *Protein J., 37*: 473-499.

Li, P; Ponnala, L; Gandotra, N; Wang, L; Si, Y; Tausta, S L; Kebrom, T H; Provart, N; Patel, R and Myers, C R (2010). The developmental dynamics of the maize leaf transcriptome. *Nat. Genet., 42*: 1060-1067.

Liscombe, D K; Ziegler, J; Schmidt, J; Ammer, C and Facchini, P J (2009). Targeted metabolite and transcript profiling for elucidating enzyme function: Isolation of novel N-methyltransferases from three benzylisoquinoline alkaloid-producing species. *Plant J., 60*: 729-743.

Low, E T L; Alias, H; Boon, S-H; Shariff, E M; Tan, C Y A; Ooi, L C; Cheah, S C; Raha, A R; Wan, K L and Singh, R (2008). Oil palm (*Elaeis guineensis* Jacq.) tissue culture ESTs: Identifying genes associated with callogenesis and embryogenesis. *BMC Plant Biol., 8*: 62. DOI: 10.1186/1471-2229-8-62.

Luo, J (2015). Metabolite-based genome-wide association studies in plants. *Curr. Opin. Plant Biol., 24*: 31-38.

Marques, J V; Dalisay, D S; Yang, H; Lee, C; Davin, L B and Lewis, N G (2014). A multi-omics strategy resolves the elusive nature of alkaloids in *Podophyllum* species. *Mol. Biosyst., 10*: 2838-2849.

Matsuda, F; Nakabayashi, R; Yang, Z; Okazaki, Y; Yonemaru, J I; Ebana, K; Yano, M and Saito, K (2015). Metabolome-genome-wide association study dissects genetic architecture for generating natural variation in rice secondary metabolism. *Plant J., 81*: 13-23.

McConnell, J R and Barton, M K (1998). Leaf polarity and meristem formation in *Arabidopsis*. *Development, 125*: 2935-2942.

Miettinen, K; Dong, L; Navrot, N; Schneider, T; Burlat, V; Pollier, J; Woittiez, L; Van Der Krol, S; Lugan, R and Ilc, T (2014). The seco-iridoid pathway from *Catharanthus roseus*. *Nat. Commun., 5*: 1-12.

Morgenthal, K; Wienkoop, S; Scholz, M; Selbig, J and Weckwerth, W (2005). Correlative GC-TOF-MS-based metabolite profiling and LC-MS-based protein profiling reveal time-related systemic regulation of metabolite-protein networks and improve pattern recognition for multiple biomarker selection. *Metabolomics, 1*: 109-120.

Morse, S M J; Shaw, G and Larner, S F (2006). Concurrent mRNA and protein from the same experimental samples using a commercial avaliable column-based RNA prearataion kit. *BioTechniques, 40*: 54-58.

Ooi, T E K; Yeap, W C; Daim, L D J; Ng, B Z; Lee, F C; Othman, A M; Appleton, D R; Chew, F T and Kulaveerasingam, H (2015). Differential abundance analysis of mesocarp protein from high-and low-yielding oil palms associates non-oil biosynthetic enzymes to lipid biosynthesis. *Proteome Sci., 13*: 28-48.

Pienkny, S; Brandt, W; Schmidt, J; Kramell, R and Ziegler, J (2009). Functional characterization of a novel benzylisoquinoline O-methyltransferase suggests its involvement in papaverine biosynthesis in opium poppy (*Papaver somniferum* L). *Plant J., 60*: 56-67.

Potters, G (2010). Systems biology of the cell. *Nat. Edu., 3*: 34.

Rai, A; Saito, K and Yamazaki, M (2017). Integrated omics analysis of specialized metabolism in medicinal plants. *Plant J., 90*: 764-787.

Ramli, U S; Chung, B L; Tahir, N; Syahanim, S; Hasliza, H; Nurazah, Z; Rozali, N; Dzulkafli, S; Ishak, N and Abrizah, O (2016). Proteomics and metabolomics: Spearheading oil palm improvement and sustainability. *Planter, 92*: 727-737.

Rischer, H; Orešič, M; Seppänen-Laakso, T; Katajamaa, M; Lammertyn, F; Ardiles-Diaz, W; Van Montagu, M C; Inzé, D; Oksman-Caldentey, K-M and Goossens, A (2006). Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus* roseus cells. *Proc. Natl. Acad. Sci. USA, 103*: 5614-5619.

Rohani, E R; Chiba, M; Kawaharada, M; Asano, T; Oshima, Y; Mitsuda, N; Ohme-Takagi, M; Fukushima, A; Rai, A and Saito, K (2016). An MYB transcription factor regulating specialized metabolisms in *Ophiorrhiza pumila*. *Plant Biotechnol. J., 33*: 1-9.

Rosli, R; Amiruddin, N; Ab Halim, M A; Chan, P-L; Chan, K-L; Azizi, N; Morris, P E; Low, E-T L; Ong-Abdullah, M and Sambanthamurthi, R (2018). Comparative genomic and transcriptomic analysis of selected fatty acid biosynthesis genes and CNL disease resistance genes in oil palm. *PLoS ONE, 13*: e0194792.

Roume, H; Heintz-Buschart, A; Muller, E E and Wilmes, P (2013). Sequential isolation of metabolites, RNA, DNA and proteins from the same unique sample. *Method. Enzymol., 531*: 219-236.

Rozali, N L; Yarmo, M A; Idris, A S; Kushairi, A and Ramli, U S (2017). Metabolomics differentiation of oil palm (*Elaeis guineensis* Jacq.) spear leaf with contrasting susceptibility to *Ganoderma boninense*. *Plant Omics, 10*: 45-52.

Saito, K (2013). Phytochemical genomics - A new trend. *Curr. Opin. Plant Biol., 16*: 373-380.

Sapcariu, S; Kanashova, T; Weindl, D; Ghelfi, J; Dittmar, G and Hiller, K (2014). Simultaneous extraction of proteins and metabolites from cells in culture. *MethodsX, 1*: 74-80.

Shearman, J R; Jantasuriyarat, C; Sangsraku, D; Yoocha, T; Vannavichit, A; Tragoonrung, S and Tangphatsomruang, S (2019). Transcriptome analysis of normal and mantled developing oil palm flower and fruit. *Genomics, 101*: 306-312.

Singh, R; Low, E-T L; Ooi, L C-L; Ong-Abdullah, M; Ting, N-C; Nagappan, J; Nookiah, R; Amiruddin, M D; Rosli, R; Manaf, M A A; Chan, K-L; Halim, M A; Azizi, N; Lakey, N; Smith, S W; Budiman, M A; Hogan, M; Bacher, B; Brunt, A V; Wang, C; Ordway, J M, Ravigadevi, S and Martienssen, R A (2013). The oil palm *SHELL* gene controls oil yield and encodes a homologue of SEEDSTICK. *Nature, 500*: 340-344.

Singh, R; Low, E-T L; Ooi, L C-L; Ong-Abdullah, M; Ting, N-C; Nagappan, J; Nookiah, R; Amiruddin, M D; Rosli, R; Manaf, M A A; Chan, K-L; Halim, M A; Azizi, N; Lakey, N; Smith, S W; Budiman. M A; Hogan, M; Bacher, B; Brunt, A V; Wang, C; Ordway, J M, Ravigadevi, S and Martienssen, R A (2014). The oil palm *VIRESCENS* gene controls fruit colour and encodes a R2R3-MYB. *Nat. Commun., 5*: 4106.

Srivastava, V; Obudulu, O; Bygdell, J; Löfstedt, T; Rydén, P; Nilsson, R; Ahnlund, M; Johansson, A; Jonsson, P and Freyhult, E (2013). OnPLS integration of transcriptomic, proteomic and metabolomic data shows multi-level oxidative stress responses in the cambium of transgenic hipl-superoxide dismutase *Populus* plants. *BMC Genomics, 14*: 893. DOI: 10.1186/ 1471-2164-14-893.

Syahanim, S; Abrizah, O; Mohamad, M A; Idris, A S and Mohd, D A (2013). Identification of differentially expressed proteins in oil palm seedlings artificially infected with *Ganoderma*: A proteomics approach. *J. Oil Palm Res., 25*: 298-304.

Tahir, N I; Shaari, K; Abas, F; Parveez, G K A; Ishak, Z and Ramli, U S (2012). Characterization of apigenin and luteolin derivatives from oil palm (*Elaeis guineensis* Jacq.) leaf using LC–ESI-MS/MS. *J. Agric. Food Chem., 60*: 11201-11210.

Tee, S-S; Tan, Y-C; Abdullah, F; Ong-Abdullah, M and Ho, C-L (2013). Transcriptome of oil palm (*Elaeis guineensis* Jacq.) roots treated with *Ganoderma boninense*. *Tree Genet. Genomes, 9*: 377-386.

Tohge, T; Wendenburg, R; Ishihara, H; Nakabayashi, R; Watanabe, M; Sulpice, R; Hoefgen, R; Takayama, H; Saito, K and Stitt, M (2016). Characterization of a recently evolved flavonol-phenylacyltransferase gene provides signatures of natural light selection in Brassicaceae. *Nat. Commun., 7*: 1-11.

Toni, L S; Garcia, A M; Jeffrey, D A; Jiang, X; Stauffer, B L; Miyamota, S D and Sucharov, C C (2016). Optimization of phenol-chloroform RNA extraction. *MethodsX, 5*: 599-608.

Udomsom, N; Rai, A; Suzuki, H; Okuyama, J; Imai, R; Mori, T; Nakabayashi, R; Saito, K and Yamazaki, M (2016). Function of AP2/ERF transcription factors involved in the regulation of specialized metabolism in *Ophiorrhiza pumila* revealed by transcriptomics and metabolomics. *Front. Plant Sci., 7*: 1-14.

Urbanczyk-Wochniak, E; Luedemann, A; Kopka, J; Selbig, J; Roessner-Tunali, U; Willmitzer, L and Fernie, A R (2003). Parallel analysis of transcript and metabolic profiles: A new approach in systems biology. *EMBO Reports, 4*: 989-993.

Valledor, L and Weckwerth, W (2014). An improved detergents-compatible gel-fractionation LC-LTQ-Orbitrap-MS workflow for plant and microbial proteomics. *Methods Mol. Biol., 1072*: 347-358.

Valledor, L; Escandón, M; Meijón, M; Nukarinen, E; Cañal, M J and Weckwerth, W (2014). A universal protocol for the combined isolation of metabolites, DNA, long RNAs, small RNAs, and proteins from plants and microorganisms. *Plant J., 79*: 173-180.

Vidal, M (2009). A unifying view of 21st century systems biology. *FEBS Letter, 583*: 3891-3894.

Vorreiter, F; Richter, S; Peter, M; Baumann, S; Von Bergen, M and Tomm, J M (2016). Comparison and optimization of methods for the simultaneous extraction of DNA, RNA, proteins and metabolites. *Anal. Biochem., 508*: 25-33.

Wang, M; Wang, P; Tu, L; Zhu, S; Zhang, L; Li, Z; Zhang, Q; Yuan, D and Zhang, X (2016). Multi-omics maps of cotton fibre reveal epigenetic basis for staged single-cell differentiation. *Nucleic Acids Res., 44*: 4067-4079.

Weckwerth, W; Wenzel, K and Fiehn, O (2004). Process for the integrated extraction, identification

and quantification of metabolites, proteins and RNA to reveal their co-regulation in biochemical networks. *Proteomics, 4*: 78-83.

Xia, W; Mason, A S; Xiao, Y; Liu, Z; Yang, Y; Lei, X; Wu, X; Ma, Z and Peng, M (2014). Analysis of multiple transcriptomes of the African oil palm (*Elaeis guineensis*) to identify reference genes for RT-qPCR. *J. Biotechnol., 184*: 63-73.

Yuan, Y (2016). Functional study of oil assembly pathway in oil palm (*Elaeis guineensis* Jacq.) fruits. Ph.D thesis, Universite de Bordeaux.

Zain, N; Seman, I; Kushairi, A and Ramli, U (2013). Metabolite profiling of oil palm towards understanding basal stem rot (BSR) disease. *J. Oil Palm Res., 25*: 58-71.

Zhou, B; Xiao, J F; Tuli, L and Ressom, H W (2012). LC-MS-based metabolomics. *Mol. Biosyst., 8*: 470-481.

Ziegler, J; Diaz-Chávez, M L; Kramell, R; Ammer, C and Kutchan, T M (2005). Comparative macroarray analysis of morphine containing *Papaver somniferum* and eight morphine free *Papaver* species identifies an O-methyltransferase involved in benzylisoquinoline biosynthesis. *Planta, 222*: 458-471.

Ziegler, J; Voigtländer, S; Schmidt, J; Kramell, R; Miersch, O; Ammer, C; Gesell, A and Kutchan, T M (2006). Comparative transcript and alkaloid profiling in *Papaver* species identifies a short chain dehydrogenase/reductase involved in morphine biosynthesis. *Plant J., 48*: 177-192.

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PUBLIC ENGAGEMENT PROMOTES CONSUMER CHOICE IN FAVOUR OF SUSTAINABLE PALM OIL

LAURA K HOBBS[1]; JOSIE W PHILLIPS[2]; AMY B STAFF[2]; AMBER GOSS[2]; LAURA FOGG-ROGERS[1] and M D FARNON ELLWOOD[3]*

ABSTRACT

Despite the superior productivity, utility and economic benefits of palm oil compared with other oil crops, the palm oil industry often receives negative publicity for its environmental impact and there is widespread confusion over the perception of palm oil. The fact is that it is difficult to avoid palm oil consumption, and consumer boycotts will do little to resolve the social and environmental issues associated with oil palm agriculture. Instead, greater awareness of the importance of certified sustainable palm oil (CSPO) is needed. We used a mixed-methods survey to explore public awareness of palm oil, and understand the factors influencing sustainable consumer choice. Our survey, conducted in the Rainforest Biome of the world-renowned Eden Project in the United Kingdom, a nation with relatively high environmental awareness, revealed that public awareness of palm oil was generally low and that consumers had poor knowledge of CSPO. We identified that the most significant barriers preventing consumer choice for CSPO products were unclear labelling, product availability and cost. We recommend that the palm oil industry focus on enhancing sustainability, promoting the benefits and increasing the visibility of CSPO in supply chains and final products, rather than waiting for consumer choice alone to drive change.

Keywords: consumer habits, oil palm, public awareness, public perception, sustainability.

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INTRODUCTION

We are living through the sixth mass extinction and human activities are destroying biodiversity at a rate significant enough for the Anthropocene to have been named after us (Pievani, 2014). Highly respected global institutions such as the United Nations (UN) and the European Parliament have declared a Climate and Ecological Emergency (European Parliament, 2019; UN Environment Programme, 2021), with public belief in the climate emergency at 81% in the United Kingdom (UK) (Flynn *et al.*, 2021). The rapid expansion of the palm oil industry has had major negative consequences for the environment, yet the industry supports the livelihoods of millions of people globally (Padfield *et al.*, 2019). To resolve this palm oil paradox, the industry must adopt and develop sustainable practices underpinning the protection of land for biodiversity, ecosystem function, carbon capture and future crop production (Corciolani *et al.*, 2019; Meijaard *et al.*, 2018).

For more than two decades academic research has considered the impact and opportunities of the palm oil industry (Padfield *et al.*, 2019), yet the

[1] Science Communication Unit, University of the West of England, Bristol, United Kingdom.

[2] Centre for Research in Biosciences, University of the West of England, Bristol, United Kingdom.

[3] School of Natural Sciences, Bangor University, Bangor, Gwynedd, LL57 2DG, United Kingdom.

* *Corresponding author e-mail: farnon.ellwood@bangor.ac.uk*

public's perception of palm oil has been influenced largely by the media (Jackson *et al.*, 2019; Yan, 2017). In 2018, the Christmas advertisement of a UK-based supermarket chain was not approved for broadcast by the non-governmental organisation (NGO) Clearcast because it was deemed too political. The advertisement, originally produced by Greenpeace, featured an animated orangutan (*Pongo* spp.) named 'Rang-tan' and the impact of oil palm expansion on its rainforest habitat. The supermarket chain had intended to use the advertisement to promote their decision to remove palm oil and its derivatives from their own-brand products. The embargo generated major publicity and discussion on social media, as well as a petition against Clearcast's decision (Mundy, 2018a). The negative response was such that Clearcast took steps to protect its staff from the backlash, including permanent removal of some social media presence (Mundy, 2018b). Celebrities and politicians used their social media platforms to call attention to palm oil and the retailer's boycott of its use (Sweney, 2018). In the week following the release of the controversial 'Rang-tan' advert (11-17 November 2018) input of the search terms 'palm oil' and 'sustainable palm oil' into the Google Search engine peaked in the UK. However, for every 100 searches using the term 'palm oil', there were just six for 'sustainable palm oil' (data extracted using Google Trends), suggesting that the spark in media attention did little to raise public awareness of sustainable palm oil, as Greenpeace had originally intended (Greenpeace, 2020).

A major challenge for producers is the perception that palm oil is an 'environmentally damaging' source of vegetable oil (Borrello *et al.*, 2019; Guadalupe *et al.*, 2019; Ostfeld *et al.*, 2019). More than 85% of the world's palm oil is produced in Indonesia and Malaysia; the industry's expansion in these countries has caused irreparable damage to Southeast Asia's primary tropical rainforest through habitat clearance (Murphy, 2014), and has reduced their carbon storage potential (Guillaume *et al.*, 2018). As production continues to expand in regions of Africa and South America we are likely to see further environmental damage (Ocampo-Peñuela *et al.*, 2018), with further loss in forest cover destroying biodiversity and undermining our ability to mitigate climate change (Guillaume *et al.*, 2018).

Oil palm expansion undoubtedly threatens biodiversity, but it has not been the sole driver of biodiversity loss in the tropics (Myzabella *et al.*, 2019; Russell, 2018). For example, fibre plantations for pulp and paper production, illegal logging and hunting have all contributed to habitat and species decline (Abood *et al.*, 2015; Jackson *et al.*, 2019; Meijaard *et al.*, 2011; Sodhi *et al.*, 2004). Similarly, the production of palm oil alternatives, which have lower yields per unit area, can be more environmentally damaging than that of palm oil (Foster *et al.*, 2011; Parsons *et al.*, 2020). Shifts in consumer choice to palm oil alternatives would therefore not necessitate a better outcome for biodiversity (Foster *et al.*, 2011; Jackson *et al.*, 2019; Meijaard *et al.*, 2018). Moreover, consumer boycotts of palm oil can have significant negative socioeconomic impacts in palm oil producing regions including the loss of employment and out-competition of smallholders by larger concessions (Lee *et al.*, 2014).

Communication campaigns and customer purchasing decisions frequently favour products which are branded 'palm oil free', and perceived as healthier, more sustainable options compared with those containing palm oil (Borrello *et al.*, 2019; Guadalupe *et al.*, 2019). However, this is not necessarily the case (Jackson *et al.*, 2019), and puts pressure on the consumer to read product labels in order to detect palm oil and its derivatives. This approach assumes a high level of awareness and time on behalf of the consumer, and also depends on the availability of products. Whilst it is possible for consumers to identify and purchase products containing certified sustainable palm oil (CSPO), cost is likely to be a barrier preventing the purchase of CSPO or palm oil-free products given that they are often more expensive (Ostfeld *et al.*, 2019).

There is evidently some way to go before the palm oil paradox is resolved, but the industry has taken major steps to develop a code of conduct under the Roundtable on Sustainable Palm Oil (RSPO) (Jackson *et al.*, 2019). The RSPO aims to establish a globally sustainable palm oil industry, currently certifying ~20% of annually produced palm oil as sustainable (RSPO, 2018a). Alongside stakeholders in the palm oil industry, the RSPO develops and implements standards for sustainable production based on ethical, transparent and legal operations, respect for human rights, support for smallholders, optimisation of productivity, efficiency, positive impacts and resilience and protection, conservation and enhancement of ecosystems and the environment (RSPO, 2018b). For example, Sime Darby, an RSPO founding-member company, cancelled planned oil palm plantations in Cameroon as establishment would have necessitated destruction of existing forest (Feintrenie, 2014).

While the majority of sustainable palm oil comes from Southeast Asia, RSPO certification is on the rise in both South America and West Africa (RSPO, 2018a). Jackson *et al.* (2019) surmised that palm oil could become the most environmentally, socially and economically sustainable vegetable oil source through adherence to the RSPO principles

and criteria. Given the potential of a globally sustainable palm oil industry, it is essential that we develop our understanding of the impact that palm oil research has on members of the public, particularly in those nations where palm oil is produced and where it is consumed. The reaction to 'Rang-tan' demonstrated how quickly an environmental campaign, intended to raise awareness of unsustainable agro-practices, can be 'hijacked' by misinformation and bias. However, impacts and opportunities for the sustainable production of oil palm vary by biogeographical region, and this requires clear communication to the public. A study by Reardon *et al.* (2019) found that consumer views of palm oil are shaped by location and can be impacted by campaigns and flows of information on palm oil. We therefore set out to establish, by way of introduction, which countries generate the most oil palm publications, before asking which research themes contribute most to those publications. We then ask, what is the level of public knowledge of palm oil in a typical consumer country, and how can consumers be encouraged to embrace sustainable palm oil?

MATERIALS AND METHODS

Literature Search, Selection Criteria and Data Acquisition

Google Scholar's global database was searched in November 2019 for original peer-reviewed research, review papers and scientific reports (excluding patents and citations) using the search term ('palm oil' or 'oil palm'). We confined the search dates between 2000, the year in which Myers *et al.* (2000) identified biodiversity 'hotspots' threatened by severe habitat loss and exploitation, and 2018, the last full year of data available at the time of searching. Cited more than 12 000 times, Myers *et al.* (2000) were the first to associate deforestation and biodiversity losses with agricultural expansion in the tropics. Our search returned 17 900 publications and consistent with Padfield *et al.* (2019), we found a near exponential increase in the number of publications per year containing the terms 'palm oil' or 'oil palm'.

To address Question 1, we randomly selected a subset of 200 publications from Google Scholar. This gave a reasonable sample size, whilst providing good resolution for analysis. We assigned each publication to a biogeographical region based on the locality of the research. These regions, all key areas of oil palm cultivation and palm oil production, were Southeast Asia, South America, Africa or Global (where the publication took a global perspective rather than being region specific). To answer Question 2, keywords were extracted from the publications and used to align each publication to one of seven research impact themes: (1) Greenhouse Gas (GHG) Emissions, (2) Biodiversity, (3) Improving Sustainability, (4) Biofuels, (5) Working Conditions and Livelihoods, (6) Production Methods and Global Trends, and (7) Deforestation. Previous work has also identified these as key themes within oil palm literature (Sheil *et al.*, 2009).

Questionnaires at the Eden Project

To address Questions 3 and 4 we performed a mixed-methods cross-sectional survey at the Eden Project. This visitor attraction, educational charity and social enterprise is located in the Southwest of England (Eden Project, 2019). Over one million people visit the Eden Project annually (Eden Project, 2018), with peak visitor flow in July and August. The visitors include education groups, local residents and tourists; 90% of visitors are from the UK, with 75% visiting while on holiday (Eden Project, 2019). During schooling periods, adult visitors predominate alongside formal education school groups, whereas families with children predominate during school holidays (Elworthy, 2016).

The Eden Project's main attraction is the Rainforest Biome, which houses the world's largest indoor tropical rainforest. It was at the centre of this indoor rainforest that we designed and built an exhibit on the story of oil palm and palm oil. Our exhibit comprises several full-size oil palms with information displaying the story of the production, impact, opportunities and sustainability of palm oil (*Figure 1*). The exhibit, which also features the RSPO logo, is in the tropical crops section of the Rainforest Biome and all visitors have to pass this section of the visitor attraction (although they do not have to read the exhibition materials). Using a convenience sampling approach, we handed questionnaires to members of the public as they passed the palm oil exhibition. A researcher handed out the paper questionnaires to consenting participants in July and August 2018. Due to high temperatures in the Rainforest Biome, the questionnaire was designed to take a maximum of 5 min to complete. As no personal data were collected, participation and the return of a completed questionnaire indicated consent for data collection. The questionnaire included open and closed questions about self-rated knowledge of palm oil, awareness of the RSPO logo, awareness of products containing palm oil, and attitudes towards sustainable palm oil consumption. Critically, we collected our data before the surge of negative attention engulfed palm oil in November 2018 following the UK supermarket chain advertisement (Sweney, 2018).

Figure 1. Collage of images from the oil palm exhibit (image credit: Eden Project).

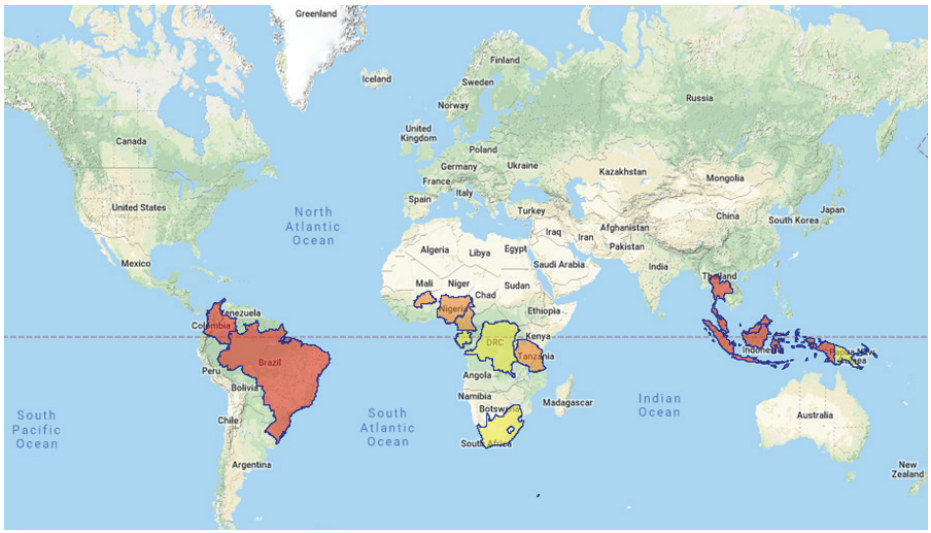

Figure 2. Map of publications by region. Red > 15 publications; Amber 5-15 publications; Green < 5 publications of our subset of 200 publications.

Statistical Analysis

After testing data for normality, we used one sample Chi-Square (χ^2) tests to assess the distribution of (a) the total number of publications by region (n=200 for all samples across Southeast Asia, South America, Africa or Global), and (b) publications by research theme. To assess the distribution of research themes within each of the different equatorial regions we used Chi-Square tests of independence (χ^2). All data collected from the Eden Project were transcribed from the questionnaire into Excel v10. A Wilcoxon signed rank test (Z) was used to compare pre- and post-exhibition self-rated knowledge of palm oil. A Spearman correlation (r_s) was used to quantify the relationship between self-rated knowledge and awareness of palm oil's use in consumable products, and a Mann-Whitney U test was used to assess awareness of the RSPO logo based on self-rated knowledge. Content analysis based on qualitative responses was used to identify the key factors that would encourage consumers to purchase CSPO products. All statistical analyses were performed in IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp. Armonk, New York, USA).

RESULTS

Question 1. Which Countries Generate the Most Oil Palm Publications?

Within the randomly selected subset of publications (n=200), oil palm was the subject of a significantly higher number of publications in Southeast Asia (n=88) and Global (n=75) than in South America (n=19) and Africa (n=18) (χ^2=81.1, df=3, p<0.01, *Figure 2*).

Question 2. Which Research Themes Contribute Most to Those Publications?

Key research themes were not distributed equally, with significantly more publications falling into 'Production methods and global trends' (n=49) and 'Working conditions and livelihood' (n=46) than would be expected for an equal distribution (25-30 publications per theme) (χ^2=46.12, df=6, p<0.01; *Figure 3*). Conversely, 'GHG emissions' (n=14) and 'Deforestation' (n=10) had significantly fewer (*Figure 3*). The under-representation of these themes was also apparent in the distribution of publications by research theme within the four geographical regions, where distribution was also significantly uneven (χ^2=0.008, df=18, p<0.05).

Question 3. What is the Level of Knowledge of Palm Oil in a Typical Consumer Country?

We collected data from 397 respondents (89% between the ages of 25-64 years old) in July and August 2018. The respondents assigned quantitative values to their knowledge of palm oil from a pre- (n=395) and post-exhibition (n=375) perspective (no knowledge = 0, some knowledge = 1, good knowledge =2, expert knowledge =3). Respondents generally rated their pre-exhibition knowledge of palm oil as poor, with a mean 'knowledge value' of 0.95 ± 0.03. However, visiting the palm oil exhibition increased this to 1.83 ± 0.02, a significant positive impact (Z=-16.13, p<0.001). *Figure 4* highlights this increase in cohort knowledge and shows that all respondents felt they had at least some knowledge of palm oil after visiting the exhibition. Complementary to this assessment, we asked respondents to indicate, from a list of commercially available products, which items they were unaware contained palm oil prior to their visit. The greatest 'unknowns' were toothpaste, bread, detergent and shampoo (*Figure 5*). When asked if they were aware of RSPO certification before their visit to the Eden Project, more than 90% (n=387) of respondents reported that they were not (*Figure 6*). Even after passing through the exhibit containing the RSPO logo, more than 80% of respondents (n=361) were unable to name the RSPO when their logo's identifying text was removed (*Figure 6*).

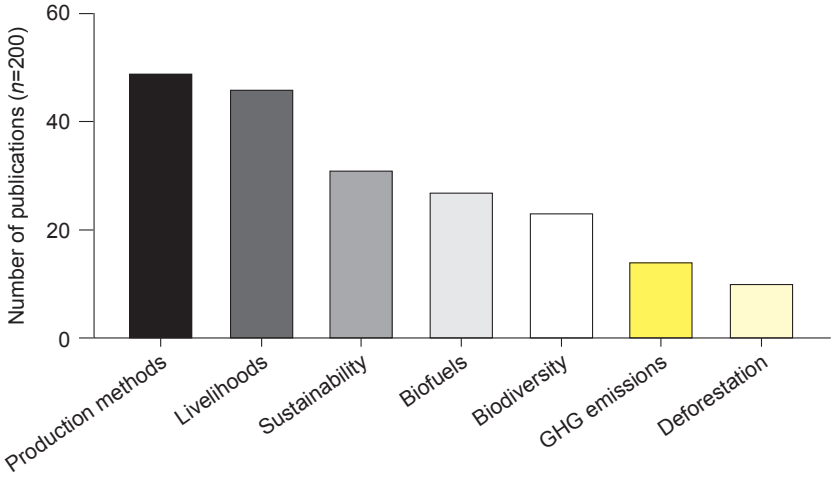

Figure 3. Distribution of publications across key research themes.

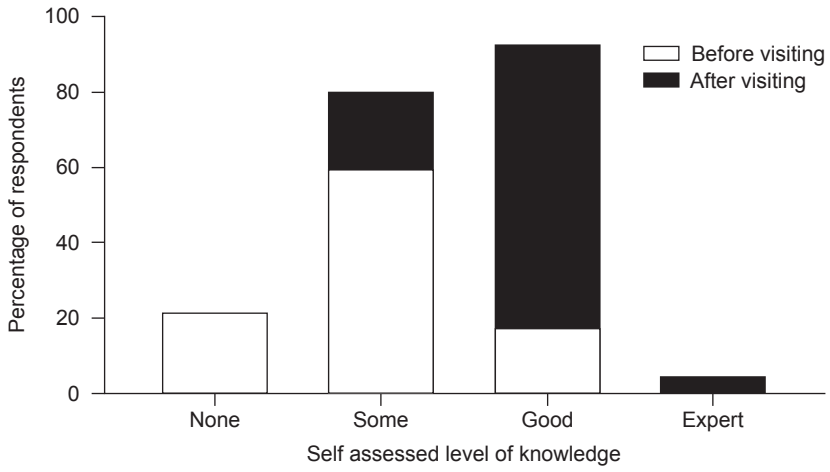

Figure 4. Change in knowledge of respondents by visiting palm oil exhibit.

Figure 5. Percentage of participants that were unaware that the product shown contained palm oil.

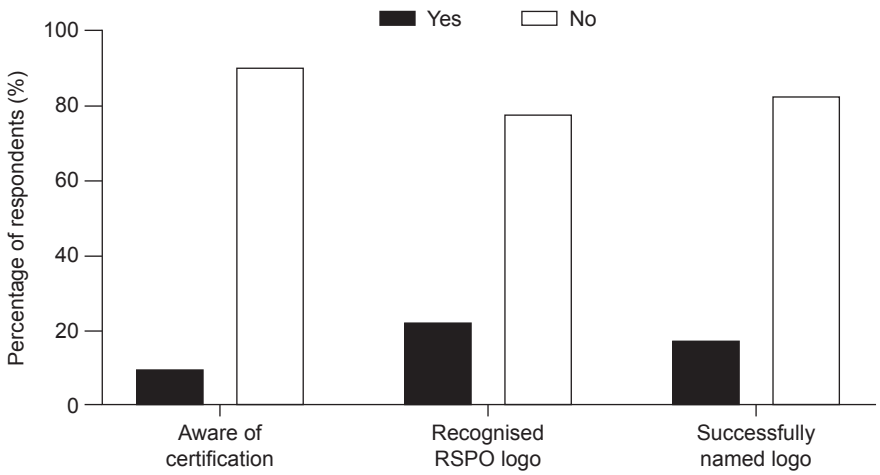

Figure 6. Percentage of participants that (a) were aware of oil palm certification, (b) claimed to recognise the RSPO logo with its text removed, and (c) successfully named the RSPO logo.

To gauge the accuracy of the respondents' self-assessment of their knowledge of palm oil, we compared their self-rated pre-exhibition knowledge with their pre-exhibition awareness of products containing palm oil. If their self-assessed knowledge was reasonably accurate, we would expect to see a negative correlation between their self-rated knowledge level, on a scale of 0-3, and the number of products that they were unaware contained palm oil. Indeed, we found a significant negative correlation (r_s=-0.371, p<0.01), indicating reasonable efficacy of respondents' self-assessment of their pre-visit knowledge levels. Similarly, respondents who rated their pre-visit knowledge as 'good' were found to be significantly more aware of the RSPO logo than those with 'some' knowledge (U=6854, p=0.002, *Figure 7*).

However, respondents' pre-visit knowledge of palm oil did not clearly align with pre-visit awareness of RSPO certification. One respondent, who reported 'expert' level pre-visit knowledge of palm oil, was not aware of RSPO certification,

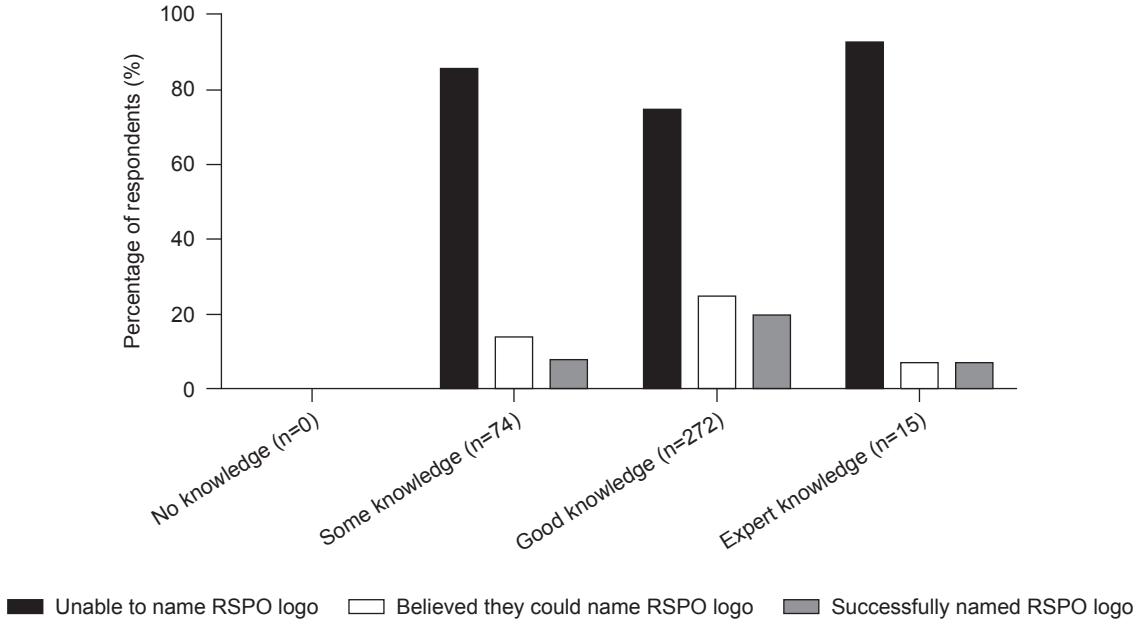

Figure 7. Percentage of participants and response to logo after engagement with the palm oil exhibit.

while ~4% of respondents who claimed to have 'no knowledge' of palm oil, reported that they were aware of RSPO certification. Generally, respondents performed poorly when asked to identify the RSPO logo (*Figure 7*). Only 21% and 7% of respondents who rated their post-visit knowledge as 'good' and 'expert' respectively were able to correctly name the RSPO logo, despite having some pre-existing awareness of CSPO and having just visited an exhibition that featured the RSPO logo.

Question 4. How Can Consumers Be Encouraged to Embrace Sustainable Palm Oil?

After visiting the Eden Project's palm oil exhibition, 78% of respondents reported that they were more likely or much more likely to buy CSPO products. Respondents identified that the most important factors that would encourage them to buy products containing CSPO were: (1) protection of primary rainforest (65.0%); (2) ensuring workers get a fair price for the palm oil they sell (25.0%); and (3) improving biodiversity on plantations (9.0%). Less than 1% of respondents stated that they 'would not buy a product containing CSPO' (0.3%) (n=312). We also assessed what factors may be preventing respondents from purchasing CSPO products and found: (1) unclear labelling (33%), (2) availability of products (24%), and (3) cost (23%) to be the most significant barriers (n=312). Upon completion of the survey, respondents were asked to 'tell us one fact about palm oil that you learnt from your visit today'. Content analysis of the responses (n=332) produced 409 individual items which were assigned across five categories: (1) the pervasiveness of palm oil (n=125, 31.0%); (2) purchasing/consumption and awareness of sustainable palm oil (n=122, 30.0%); (3) biodiversity/environmental issues (n=86, 21.0%); (4) production and yield (n=74, 18.0%) and (5) other (n=2, 0.5%).

DISCUSSION

Our aim was to explore public awareness of palm oil and RSPO certification and to understand how the palm oil industry can assist consumers in making informed and sustainable choices. This is important, as gaining a better understanding of how to harness consumer purchasing power will be key to driving sustainability further up the global agenda. In line with Padfield *et al*. (2019), we found that the number of peer-reviewed palm oil related publications has increased almost exponentially since the year 2000. With the majority of these publications focussed on Southeast Asia, comparatively few focused on oil palm agriculture in Africa and South America. This is perhaps not surprising given that Southeast Asia's commercial oil palm cultivation boom began shortly after Malaysia's independence more than 60 years ago (Murphy, 2014), whereas oil palm expansion in other regions has largely taken place since 2000 (Carrere, 2013; Pardo Vargas *et al*., 2015). Thus, the disparity in the number of palm oil publications across production regions that we observed was likely a fair representation of the distribution of global palm oil research within the academic literature. While research conducted in Southeast Asia has been largely reactive to oil palm expansion and its impacts, the likely gaps in

knowledge arising from the gap in research from other growing regions present an opportunity for proactive research and clear communication of the benefits of sustainable palm oil. By applying knowledge gained through oil palm development in Southeast Asia to other growing regions in West Africa and South America, the global palm oil industry could vastly improve its sustainability. The reaction to the 'Rang-tan' campaign demonstrates how public outrage at unsustainable agro-practices can spur consumer boycotting, but it also highlights the power of campaigning through storytelling. For example, in order to address the negative association between palm oil and the decline of the orangutan, there is an urgent need to deliver positive stories from Southeast Asia. Furthermore, communication of positive and innovative research in areas of new development, where there is still significant opportunity to develop truly sustainable palm oil practices, is of the utmost importance. For example, a recent study in Colombia showed that where oil palm plantations replaced pasture, carbon losses were reduced by 99.7 ± 9.6% when compared to rainforest conversion (Quezada *et al.*, 2019), thereby increasing the carbon sequestration potential of the landscape and sparing endemic-species-rich forest ecosystems (Ocampo-Peñuela *et al.*, 2018; Prescott *et al.*, 2016).

Publications were assigned to research categories using a 'best-fit' method; much of the research categorised showed some overlap between definitive themes. These overlaps were most common for ecological and environmental categories where, for example, it would have been feasible to assign a publication to either the Deforestation or Biodiversity category. Nevertheless, we found ecological and environmental research (*i.e.*, Biodiversity, Deforestation and GHG emissions) to be under-represented in the literature compared with publications that considered the social (*i.e.*, working conditions and livelihoods) and economic (*i.e.*, production methods and global trends) impacts and opportunities of oil palm. This suggests that more oil palm research has been conducted from an anthropocentric, rather than an ecocentric perspective. Padfield *et al.* (2019) made similar observations, and noted that peer-reviewed articles containing the terms 'palm oil' or 'oil palm' were heavily weighted towards engineering and biofuel topics. Topics such as land use change and biodiversity were far less common. Although landmark publications such as Myers *et al.* (2000) have highlighted the ecological consequences of intense anthropogenic activity, oil palm expansion has continued to drive deforestation and losses to biodiversity (Wilcove *et al.*, 2013). Disparities in the distribution of publications by research theme may indicate that the ecological and environmental impacts of the palm oil industry are less of a research priority than social and economic impacts. However, given that research is underpinned by funding availability, this could also suggest that funding, and especially industrial funding, favours socio-economic (*i.e.*, anthropocentric) over ecological and environmental (*i.e.*, ecocentric) research. This is concerning because as production expands in South America and Africa, where much of the population lives below the poverty line (World Bank, 2019), socio-economic research and development is likely to be prioritised over that of conservation (Billé *et al.*, 2012).

Our study, in line with previous work by Padfield *et al.* (2019), revealed a substantial volume of research into palm oil sustainability. Whilst this may highlight an historical disregard of sustainability (Morgans *et al.*, 2018), it is potentially indicative of a shift in the industry's priorities in favour of sustainable development (Padfield *et al.*, 2019). This is evidenced by the fact that a non-trivial component (~20%) of palm oil produced globally is now certified by the RSPO (Roundtable on Sustainable Palm Oil, 2018a). However, negative media attention and product boycotting have often drawn attention away from the benefits of CSPO and efforts to promote its production (Jackson *et al.* 2019; Laurance *et al.*, 2010). For this reason, we designed the palm oil exhibition in the Rainforest Biome at the Eden Project to provide the public with a balanced narrative of the scientific evidence on oil palm agriculture.

Our survey at the Eden Project provided a good insight into the public's awareness of palm oil before the 'Rang-tan' advertising campaign went viral, and an opportunity to understand the effectiveness of the exhibition as a platform for public engagement of a complex socio-economic and environmental issue. Our results indicated that engagement with the exhibit content had a significant and positive impact on respondents' knowledge of palm oil and its products. Participants generally had poor knowledge of palm oil and its use in consumer products, with less than 20% of respondents reporting to have had a 'good knowledge' of palm oil before visiting the exhibition (*Figure* 4). This demonstrates that despite an exponential increase in palm oil research, a disconnect remains between academic research and public awareness of palm oil. Thus, exhibitions such as ours at the Eden Project will become an increasingly important tool for addressing the challenge of convincing consumers to buy CSPO (Laurance *et al.*, 2010). Awareness of palm oil in consumer products varied by product type, and we found that respondents were most likely to be unaware that personal care and household products such as toothpaste (70% unaware) and laundry detergent (53% unaware) contained palm oil. Consumers were far less likely to be unaware of palm oil's prevalence in food products such

as margarine (18% unaware) and biscuits (27% unaware), and this is likely to be a response to palm oil's portrayal in the media which has frequently focused on demand from the food industry (Jackson *et al.*, 2019). After visiting the exhibit, none of the respondents reported 'no knowledge' of palm oil. Thus, our results provide further evidence of the effectiveness of scientific exhibits for engaging consumers and improving awareness of complex environmental issues.

A study by Ostfeld *et al.* (2019) revealed that recognition of the RSPO's logo was effectively zero, and thus, recommended that government policies should be amended to require companies to source 100% CSPO instead of relying on consumers to demand and purchase products containing CSPO. We similarly observed a near complete inability of participants to name the RSPO logo, even after visiting the exhibition, which clearly displays the RSPO logo. This suggests that simply displaying the RSPO 'ecologo' is not enough and will not be sufficient to encourage a change in the buying habits of consumers. This can be remedied, as other ecolabels are widely recognised; for example the Fairtrade logo was recognised by 82% of shoppers in the UK (Ostfeld *et al.*, 2019). Though Fairtrade was established over 25 years ago, its reputability has been underpinned by extensive outreach work, advertising and marketing in the mainstream media (Fairtrade Foundation, 2019). At present, the RSPO logo is rarely used as a consumer-facing label, thus, may not be considered an immediately applicable tool for engaging consumers (Ostfeld *et al.*, 2019). Therefore, efforts from the RSPO and its member companies to increase visibility within the mainstream media in countries that are major consumers of palm oil would likely pay dividends in terms of public understanding, RSPO logo recognition and willingness to support CSPO.

Indeed, we found that the Eden Project's palm oil exhibit had a major and positive impact on the willingness of visitors to support CSPO, with 78% of respondents reporting that they were more likely or much more likely to buy CSPO products after visiting the exhibit. This clearly evidences the positive role that tourism attractions and botanical gardens can play in raising awareness and changing attitudes towards environmental issues. There is much evidence that tourists value the environment and with targeted, relevant communications, could be encouraged towards more sustainable consumption behaviour (Font and McCabe, 2017). While visitors to attractions such as the Eden Project could be considered a key audience for CSPO products, efforts must also be made to ensure that learning opportunities for improved awareness of CSPO as well as access to products are available to audiences beyond those who would visit an educational charity and visitor destination.

Despite finding that palm oil publications regarding 'Biodiversity' and 'Deforestation' were under-represented in comparison with other themes in the literature, when we asked visitors at the Eden Project what would encourage them to purchase CSPO, 'protection of primary rainforest' was found to be of the highest priority (65%). When respondents were asked to relay one fact about palm oil that they had learnt from their visit, the 'pervasiveness of palm oil' (31%) as well as 'awareness of sustainable palm oil' (30%) were most common responses. This contrasts with the broad unawareness of the presence of palm oil in different product groups that respondents reported before visiting the exhibition. Reported barriers to purchasing CSPO did not indicate a lack of interest in or willingness to support CSPO; we identified that unclear labelling, lack of product availability and cost were the key factors inhibiting consumer choice. The responses provided in our survey of consumers were generally consistent with those reported from palm oil industry stakeholders by Padfield *et al.* (2019). Though our respondents did not explicitly state that protecting biodiversity was the most important factor that would encourage consumers to purchase CSPO products, the protection of rainforest will certainly serve to protect biodiversity. Together, this suggests that while consumers are concerned with the prevalence of palm oil in products, the use of positive messages such as the protection of rainforest or fair prices for workers will be key to encouraging consumers to make sustainable purchasing decisions rather than boycotting palm oil altogether. This should alleviate fears from manufacturers and retailers over drawing attention to the fact that they are using palm oil (Chaudhari and Purkayastha, 2011; Ostfeld *et al.*, 2019), and provide direction for future marketing and campaigning decisions. In addition, this finding can inform improvement of the RSPO's operations. The roundtable has attracted criticism for ineffective monitoring and its failure to halt the destruction of primary rainforest and provide beneficial ecological outcomes for its approved members (Morgans *et al.*, 2018; Schouten and Glasbergen, 2011). Thus, prioritisation of rainforest conservation, paired with effective communication of this through campaigns and exhibitions, may increase support and demand for certified palm oil.

Awareness in botanical gardens and other relevant settings can have a measurable impact on awareness and knowledge of palm oil and the issues surrounding it, but this is not the only form of communication that is needed to effect change. Environmentalism is a fast-moving field, with foci often changing with each new 'crisis' reported (Goldsmith and Goldsmith, 2011). Longer-term engagement with relevant information is needed, particularly in a time when 'fake news' and viral

online content can rapidly disseminate facts and information which may have a negative impact on the environment and wildlife, however unintended (Clarke *et al.*, 2019). The role of scientists should be dualistic in nature, working towards engaging the public in discussion while supporting the development and implementation of sustainable practices. In the UK, the success of a collaborative approach is demonstrated by Chester Zoo's 'Sustainable Palm Oil City' initiative, which has led to Chester becoming the first sustainable palm oil city in the world. The campaign, alongside increasing consumer awareness, assisted more than 50 organisations including manufacturers, restaurants, cafes, and educational institutions to audit their supply chains and make a time-bound commitment to using 100% RSPO certified palm oil (Chester Zoo, 2019a). Through providing a toolkit, educational resources, and an incentive for local businesses, Chester Zoo expanded its reach to new audiences (Ancrenaz *et al.*, 2018; Chester Zoo, 2019b). They also addressed some of the key barriers to sustainable palm oil consumption identified by our study, such as unclear labelling and lack of availability. Similar initiatives are now under development in Bristol, Newquay, and Oxford (Bristol Zoo, 2018; Chester Zoo, 2019c). Increasing public awareness is key to improving the reputation of sustainable palm oil, and cross-organisational collaboration will allow stakeholders throughout the supply chain to feel confident in promoting what has become an essential ingredient to 21st century life.

CONCLUSION

Consumer awareness of palm oil and its prevalence in products remains low in the UK, as does knowledge of CSPO, and the RSPO. However, consumers will support rather than boycott products which protect rainforests, which should encourage manufacturers to promote their use of CSPO. This will be important in driving change, but the palm oil industry needs to do more to increase awareness of CSPO. Visitor attractions and educational charities such as the Eden Project represent an effective opportunity to support public engagement and raise awareness of the complex underlying issues and the viability of CSPO as a solution. Public perception of the palm oil industry could be improved through further outreach work and positive storytelling led by un-biased parties.

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REFERENCES

Abood, S A; Lee, J S H; Burivalova, Z; Garcia-Ulloa and Koh, J L P (2015). Relative contributions of the logging, fibre, oil palm, and mining industries to forest loss in Indonesia. *Conserv. Lett.*, *8*: 58-67. DOI: 10.1111/conl.12103.

Ancrenaz, M; Barton, C; Riger, P and Wich, S (2018). Building relationships: How zoos and other partners can contribute to the conservation of wild orangutans *Pongo* spp. *Int. Zoo. Yb.*, *52(1)*: 164-172.

Billé, R; Lapeyre, R and Pirard, R (2012). Biodiversity conservation and poverty alleviation: A way out of the deadlock? *Surveys and Perspectives Integrating Environment and Society*, *5(1)*: 1-15.

Borrello, M; Annunziata, A and Vecchio, R (2019). Sustainability of palm oil: Drivers of consumers' preferences. *Sustainability*, *11*: 4818. DOI: 10.3390/su11184818.

Bristol Zoo (2018). Palm oil: Promoting sustainable palm oil. https://bristolzoo.org.uk/save-wildlife/conservation-and-research/palm-oil-project, accessed on 27 April 2021.

Carrere, R (2013). *Oil Palm in Africa: Past, Present and Future Scenarios.* World Rainforest Movement. 79 pp.

Chaudhari, A and Purkayastha, D (2011). Greenpeace, Nestlé and the palm oil controversy: Social media driving change? Report 911-010-1. IBS Centre for Management Research. 24 pp.

Chester Zoo (2019a). Chester named world's first sustainable palm oil city. https://www.chesterzoo.org/whats-happening/zoo-news/2019/03/chester-named-worlds-first-sustainable-palm-oil-city, accessed on 27 April 2021.

Chester Zoo (2019b). Our sustainable palm oil challenge. https://www.chesterzoo.org/what-you-can-do/our-campaigns/sustainable-palm-oil/, accessed on 27 April 2021.

Chester Zoo (2019c). UK cities and towns join sustainable palm oil city movement. https://www.chesterzoo.org/news/uk-cities-and-towns-join-sustainable-palm-oil-city-movement/, accessed on 27 April 2021.

Clarke, T A; Reuter, K E; LaFleur, M and Schaefer, M S (2019). A viral video and pet lemurs on Twitter. *PLoS ONE*, *14(1)*: e0208577. DOI: 10.1371/journal.pone.0208577.

Corciolani, M; Gistri, G and Pace, S (2019). Legitimacy struggles in palm oil controversies: An institutional perspective. *J. Clean. Prod.*, *212(1)*: 1117-1131.

Eden Project (2018). Eden Project Annual Review 2017/2018. https://www.edenproject.com/eden-story/about-us/annual-and-sustainability-reports, accessed on 9 October 2019.

Eden Project (2019). Admission. https://www.edenproject.com/buy-tickets-0, accessed on 9 October 2019.

Elworthy, J (2016). Eden Project palm oil exhibit proposal to University of the West of England (UWE). Market research data. Unpublished.

European Parliament (2019). The European Parliament declares climate emergency. https://www.europarl.europa.eu/news/en/press-room/20191121IPR67110/the-european-parliament-declares-climate-emergency, accessed on 27 April 2021.

Fairtrade Foundation (2019). The history of Fairtrade. https://www.fairtrade.org.uk/What-is-Fairtrade/The-impact-of-our-work/The-History-of-Fairtrade, accessed on 30 January 2020.

Feintrenie, L (2014). Agro-industrial plantations in Central Africa, risks and opportunities. *Biodivers. Conserv.*, *23(6)*: 1577-1589.

Flynn, C; Yamasumi, E; Fisher, S; Snow, D; Grant, Z; Kirby, M; Browning, P; Rommerskirchen, M and Russell, R (2021). People's Climate Vote; Results. UNDP and University of Oxford. 68 pp.

Font, X and McCabe, S (2017). Sustainability and marketing in tourism: Its contexts, paradoxes, approaches, challenges and potential. *J. Sustain. Touri.*, *25(7)*: 869-883.

Foster, W A; Snaddon, J L; Turner, E C; Fayle, T M; Cockerill, T D; Ellwood, M D F; Broad, G R; Chung, A Y C; Eggleton, P; Vun Khen, C and Yusah, K M (2011). Establishing the evidence base for maintaining biodiversity and ecosystem function in the oil palm landscapes of South East Asia. *Phil. Trans. R. Soc. B*, *366*: 3277-3291.

Goldsmith, E B and Goldsmith, R E (2011). Social influence and sustainability in households. *Int. J. Consum. Stud.*, *35*: 117-121.

Greenpeace (2020). Palm oil. https://www.greenpeace.org.uk/challenges/palm-oil/, accessed on 30 January 2020.

Guadalupe, G; Lerma-García, M; Fuentes, A; Barat, J; Bas, M and Fernández-Segovia, I (2019). Presence of palm oil in foodstuffs: Consumers' perception. *Br. Food J.*, *121(9)*: 2148-2162. DOI: 10.1108/BFJ-09-2018-0608.

Guillaume, T; Kotowska, M M; Hertel, D; Knohl, A; Krashevska, V; Murtilaksono, K; Scheu, S and Kuzyakov, Y (2018). Carbon costs and benefits of Indonesian rainforest conversion to plantations. *Nat. Commun.*, *9*: 2388. DOI: 10.1038/s41467-018-04755-y.

Jackson, T A; Crawford, J W; Traeholt, C and Sanders, T A B (2019). Learning to love the world's most hated crop. *J. Oil Palm Res.*, *31(3)*: 331-347.

Laurance, W F; Koh, P K; Butler, R; Sodhi, N S; Bradshaw, C J; Neidel, D; Consunji, H and Vega, J M (2010). Improving the performance of the roundtable on sustainable palm oil for nature conservation. *Conserv. Biol.*, *24(2)*: 377-381.

Lee, J S H; Garcia-Ulloa, J; Ghazoul, G; Obidzinski, K and Pin Koh, L (2014) Modelling environmental and socio-economic trade-offs associated with land-sparing and land-sharing approaches to oil palm expansion. *J. Appl. Ecol.*, *51(5)*: 1366-1377.

Meijaard, E; Buchori, D; Hadiprakarsa, Y; Utami-Atmoko, S S; Nurchahyo, A; Tjiu, A; Prasetyo, D; Nardiyono, Christie, L and Ancrenaz, M (2011). Quantifying killing of orangutans and human-orangutan conflict in Kalimantan, Indonesia. *PLoS ONE, 6*: e27491. DOI: 10.1371/journal.pone.0027491.

Meijaard, E; Garcia-Ulloa, J; Sheil, D; Wich, S A; Carlson, K M; Juffe-Bignoli, D and Brooks, T M (2018). Oil palm and biodiversity. A situation analysis by the IUCN oil palm task force. IUCN Oil Palm Task Force Gland, Switzerland. 116 pp.

Morgans, C; Meijaard, E; Santika, T; Law, E; Budiharta, S; Ancrenaz, M and Wilson, K (2018). Evaluating the effectiveness of palm oil certification in delivering multiple sustainability objectives. *Environ. Res. Lett.*, *13(6)*: 064032. DOI: 10.1088/1748-9326/aac6f4.

Mundy, C (2018a). Response to coverage of decision not to clear the Iceland ad. https://www.clearcast.

co.uk/blog/clearcasts-md-responds-to-coverage-of-their-decision-not-to-clear-the-iceland-ad/, accessed on 9 October 2019.

Mundy, C (2018b). Clearcast's MD speaks out on abuse directed at staff following Iceland decision. https://www.clearcast.co.uk/blog/clearcasts-md-speaks-out-on-abuse-following-iceland-decision/, accessed on 9 October 2019.

Murphy, D J (2014). The future of oil palm as a major global crop: Opportunities and challenges. *J. Oil Palm Res.*, *26(1)*: 1-24.

Myers, N; Mittermeier, R; Mittermeier, C; da Fonseca, G and Kent, J (2000). Biodiversity hotspots for conservation priorities. *Nature, 403(6772)*: 853-858.

Myzabella, N; Fritschi, L; Merdith, N; El-Zaemey, S; Chih, H J and Reid, A (2019). Occupational health and safety in the palm oil industry: A systematic review. *Int. J. Occup. Environ. Med.*, *10(4)*: 159-173.

Ocampo-Peñuela, N; Garcia-Ulloa, J; Ghazoul, J and Etter, A (2018). Quantifying impacts of oil palm expansion on Colombia's threatened biodiversity. *Biol. Conserv.*, *224*: 117-121.

Ostfeld, R; Howarth, D; Reiner, D and Krasny, P (2019). Peeling back the label-exploring sustainable palm oil ecolabelling and consumption in the United Kingdom. *Environ. Res. Lett.*, *14*: 014001. DOI: 10.1088/1748-9326/aaf0e4.

Padfield, R; Hansen, S; Davies, Z G; Ehrensperger, A; Slade, E M; Evers, S; Papargyropoulou, E; Bessou, C; Abdullah, N and Page, S (2019). Co-producing a research agenda for sustainable palm oil. *Front. Glob. Change*, *2*. DOI: 10.3389/ffgc.2019.00013.

Pardo Vargas, L E; Laurance, W F; Clements, G R and Edwards, W (2015). The impacts of oil palm agriculture on Colombia's biodiversity: What we know and still need to know. *Trop. Conserv. Sci.*, *8(3)*: 828-845.

Parsons, S; Raikova, S and Chuck, C J (2020). The viability and desirability of replacing palm oil. *Nat. Sustain.*, *3*: 412-418. DOI: 10.1038/s41893-020-0487-8.

Pievani, T (2014). The sixth mass extinction: Anthropocene and the human impact on biodiversity. *Rend. Fis. Acc. Lincei.*, 25: 85-93. DOI: 10.1007/s12210-013-0258-9.

Prescott, G; Gilroy, J; Haugaasen, T; Medina Uribe, C; Foster, W A and Edwards, D (2016). Reducing the impacts of Neotropical oil palm development on functional diversity. *Biol. Conserv*. p. 139-145.

Quezada, J; Etter, A; Ghazoul, J; Buttler, A and Guillaume, T (2019). Carbon neutral expansion of oil palm plantations in the Neotropics. *Sci. Adv.*, *5(11)*: eaaw4418. DOI: 10.1126/sciadv.aaw4418.

Reardon, K; Padfield, R and Salim, H K (2019). Consumers don't see tigers dying in palm oil plantations: A cross-cultural comparative study of UK, Malaysian and Singaporean consumer views of palm oil. *Asian Geogr., 36(2)*: 117-141. DOI: 10.1080/10225706.2019.1621187.

Roundtable on Sustainable Palm Oil (2018a). RSPO Impact Report 2018. Impacts and Evaluation Division, RSPO Secretariat, Kuala Lumpur, Malaysia. 86 pp.

Roundtable on Sustainable Palm Oil (2018b). Principles and criteria for the production of sustainable palm oil 2018. Roundtable on Sustainable Palm Oil, Kuala Lumpur, Malaysia. 137 pp.

Russell, M (2018). Palm oil: Economic and environmental impacts. *European Parliamentary Research Service*. https://epthinktank.eu/2018/02/19/palm-oil-economic-and-environmental-impacts/, accessed on 9 October 2019.

Schouten, G and Glasbergen, P (2011). Creating legitimacy in global private governance: The case of the Roundtable on Sustainable Palm Oil. *Ecol. Econ., 70(11)*: 1891-1899.

Sheil, D; Casson, A; Meijaard, E; van Nordwijk, M; Gaskell, J; Sunderland-Groves, J; Werts, K and Kanninen, M (2009). The impacts and opportunities of oil palm in Southeast Asia: What do we know and what do we need to know? Centre for International Forestry Research, Bogor, Indonesia.

Sodhi, N S; Koh, L P; Brook, B W and Ng, P K L (2004). Southeast Asian biodiversity: An impending disaster. *Trends Ecol. Evol.*, *19(12)*: 654-660.

Sweney, M (2018). Iceland to let loose animatronic orangutan after Christmas ad ban. The Guardian. https://www.theguardian.com/business/2018/nov/14/iceland-let-loose-animatronic-orangutan-after-christmas-advert-ban-palm-oil, accessed on 13 November 2019.

UN Environment Programme (2021). Facts about the climate emergency. https://www.unep.org/explore-topics/climate-change/facts-about-climate-emergency, accessed on 27 April 2021.

Wilcove, D S; Giam, X; Edwards, D P; Fisher, B and Koh, L P (2013) Navjot's nightmare revisited: Logging, agriculture and biodiversity in Southeast Asia. *Trends Ecol. Evol.*, *28(9)*: 531-540.

World Bank (2019). *Poverty and Inequality: Featured Indicators (Poverty headcount ratio at national poverty lines).* http://datatopics.worldbank.org/world-development-indicators/themes/poverty-and-inequality.html#featured-indicators_1, accessed on 13 November 2019.

Yan, N W (2017). A makeover for the world's most hated crop. *Nature*, *543*: 306-308. DOI: 10.1038/543306a.

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IDENTIFICATION OF OIL PALM ROOT-SPECIFIC GENES THROUGH MINING OF RNA-SEQ DATA AND RT-qPCR ANALYSIS

SUBHI SITI MASURA[1,2,*]; OMAR ABD RASID[1]; NOOR AZMI SHAHARUDDIN[2, 3]; MAT YUNUS ABDUL MASANI[1]; KUANG-LIM CHAN[1]; ENG-TI LESLIE LOW[1]; SITI SURIAWATI BADAI[1]; AYUB NOR HANIN[1]; ABDUL RAHMAN SITI RAHMAH[1]; MOHD PUAD ABDULLAH[2]; AZZREENA MOHAMAD AZZEME[2] and GHULAM KADIR AHMAD PARVEEZ[1]

ABSTRACT

Identification of novel genes that are specifically expressed in root is essential for isolation and characterisation of root-specific promoters. Mining the transcriptome of various oil palm tissue-specific data generated from ribonucleic acid-sequencing (RNA-Seq) technology has enabled the discovery of root-specific genes. A total of seven candidates of root-specifically or preferentially expressed genes were selected from RNA-Seq analysis, and the gene expression profiles were validated using real-time quantitative polymerase chain reaction (RT-qPCR). The relative fold change of transcript expression in RT-qPCR was statistically analysed by comparing with root tissues at the in vitro *culture stage (RS1). Results showed that the transcript annotated as an oil palm metallothionine (*EgMT*) gene was significantly upregulated at around 7 to 170-fold across the different developmental stages of root tissues. A proline-rich protein* (EgPRP1) *transcript was also significantly upregulated by about 7 to 55-fold. Both* EgMT *and* EgPRP1 *transcripts had relatively low expressions in the other tissues studied. The high levels of expression of* EgMT *and* EgPRP1 *in roots highlighted the genes' promoter's potential to regulate a strong expression level of transgenes in a root-specific manner.*

Keywords: *Elaeis guineensis*, root-specific genes, RNA-Seq, RT-qPCR.

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INTRODUCTION

The study of gene expression profiles of cells and tissues using transcriptome data is essential in identifying novel genes. Transcriptome is a complete set of transcripts, including both coding and non-coding ribonucleic acids (RNAs) in a specific type of cell or tissue. Since the last decade, numerous technologies based on hybridisation and sequence-based methods have been developed for generating and quantifying transcriptome. With the advancement of the sequencing method, RNA sequencing (RNA-Seq) technology has emerged as one of the most potent transcript profiling techniques available to date. RNA-Seq provides a more accurate measurement of gene expression and enables the discovery of gene isoforms. Besides, RNA-Seq can also be used to investigate splice sites, alternatively spliced isoforms, small and non-coding RNA (Rivas *et al.,* 2011), single nucleotide polymorphism and post-transcriptional modification (Lalonde *et al.,* 2011). This technology

[1] Malaysian Palm Oil Board,
6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.

[2] Department of Biochemistry,
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia, 43400 UPM Serdang,
Selangor, Malaysia.

[3] Institute of Plantation Studies,
Universiti Putra Malaysia, 43400 UPM Serdang,
Selangor, Malaysia.

* *Corresponding author e-mail: masura@mpob.gov.my*

offers a rapid and comprehensive transcript profiling technique with considerably less time and a lower cost (Alpern *et al.*, 2019; Lister *et al.*, 2008; Oikonomopoulos *et al.*, 2020).

The RNA-Seq technology has been applied in numerous crops, including oil palm. As a high yielding source of vegetable oil, palm oil is widely consumed for edible purposes and used as feedstock for oleochemicals and biofuels production. Palm oil accounts for about one-third of world vegetable oil consumption (Kushairi *et al.*, 2018), and the demands will continue to increase due to the growth of the world's population. The effort to genetically engineer oil palm with aims to improve its oil for different oil quality or higher oil yield has been pioneered by the Malaysian Palm Oil Board (MPOB) since the mid-90s (Masani *et al.*, 2018; Masura *et al.*, 2017; Parveez *et al.*, 2015; Rasid *et al.*, 2020). MPOB has reported a breakthrough in oil palm research by deciphering the oil palm genome sequence of the *Pisifera* fruit form of *Elaeis guineensis* (Singh *et al.*, 2013). A total of ~1.5 Gb sequences of the 1.8 Gb genome with the size of 1.05 Mb were released to the public domain (Low *et al.*, 2017; Singh *et al.*, 2013). Transcriptome data were also generated, including those from leaf, inflorescence, pollen, mesocarps, kernel, roots and shoot (http://genomsawit.mpob.gov.my), and this endeavour aids the effort in discovering novel genes of important traits in various tissues of interest, including root.

Protection against drought, increased tolerance to salt, nutrients uptake and increased resistance to pests and diseases are among the useful traits that can be produced through modification of root systems (Li *et al.*, 2019; Potenza *et al.*, 2004). In oil palm, modification of the root system has been focused on producing plant resistance to diseases, particularly basal stem rot that has caused serious economic losses to the oil palm industry. The disease is caused by *Ganoderma boninense* fungus that develops from airborne spores and spreads in the soil through the root (Naher *et al.*, 2013). The adoption of genetic engineering is one of the biotechnological approaches to control or eradicate the spread of the disease. Targeting the expression of fungal resistant genes in oil palm roots could increase the plant defence system against the pathogen. In addition to the disease, the oil palm industry is also to anticipate the effects of climate change in the future that will result in a decline in crude palm oil production (Kushairi *et al.*, 2017). It will likely continue to influence soil properties, which may affect nutrient uptake by the palms (Rival, 2017). Therefore, the improvement of root traits through genetic engineering especially to maximise nutrients and water uptake, would increase crop yields, particularly in unfavourable environments such as under water shortage and low nutrient soils (Meister *et al.*, 2014; Wasson *et al.*, 2012).

To target genetic modification in the root, promoters or regulatory regions that regulate the expression of transgenes in a root-specific manner are required. Several plant root-specific promoters have been isolated and functionally characterised, including *TobRB7, Pyk10, RCc3, PsPR10, MsPRP2, GmPRP2, OsGRP7, Os03g01700* and *Os02g37190* (Chen *et al.*, 2014; Jeong *et al.*, 2010; Liu and Ekramoddoullah, 2003; Nitz *et al.*, 2001; Xue *et al.*, 2016; Yamamoto *et al.*, 1991). However, many studies have shown that the promoter efficiencies in the heterologous system vary considerably, probably due to the absence of some factors essential for promoter regulation (Hernandez-Garcia and Finer, 2014). In oil palm, two root inducible promoters, derived from metallothionine (*MT3-B*) and phosphate transporter (*EgPHT1*) genes have been isolated and characterised (Ahmadi *et al.*, 2018; Zubaidah and Siti Nor Akmar, 2005). *MT3-B* promoter's activity was induced by the presence of metal ions, while *EgPHT1* was induced under inorganic phosphate (Pi) deficiency. Although the strong inducible promoters can be of great benefit, this characteristic could limit their uses as root-specific promoters. As plants have several thousand genes with a vast range of functionalities, it is not surprising that an astonishingly high number of promoters and regulatory elements remain to be discovered (Hernandez-Garcia and Finer, 2014). This effort would lead to significant improvement in the regulation of numerous phenotypes and transgene expression, since a wide range of promoters is available for extensive genetic engineering works.

Prior to promoter isolation, the foremost prerequisite study is to identify genes that are specifically or preferentially expressed in the tissue of interest. The availability of oil palm transcriptome data generated from different tissues has enabled various analyses to be performed, including RNA-Seq analysis. The strategy allows quantification of differentially expressed gene, resulting in discovery of novel genes beneficial for genetic engineering work, including promoter isolation. This study identified two root-specific promoter candidates from the transcriptome data through the RNA-Seq analysis and real-time quantitative polymerase chain reaction (RT-qPCR) analyses. To our knowledge, this is the first study to identify oil palm root-specific or preferentially expressed genes through the mining of oil palm transcriptome data. The discovery would lead to the possession of a well-furnished toolbox of promoters necessary for gene stacking technologies to address more complex agronomic traits.

MATERIALS AND METHODS

Plant Materials

All samples used for RNA isolation were derived from *Elaeis guineensis* (*Tenera*). These include tissues from roots (at different developmental stages), mesocarp, kernel, green leaves, young leaves, inflorescences (male and female), callus, cabbage and plantlets. All samples were collected from an oil palm elite planting material, namely P456 clone. The P456 is a reclone of P164, an MPOB standard clone that produces high oil yield (8-10 t ha^{-1} yr^{-1}). The clone also has high success rates in tissue culturing and meagre mantling rates in the field (Zulkifli *et al.*, 2017).

RNA-Seq Analysis

To identify candidate genes specifically or preferentially expressed in roots, a total of 144 oil palm transcriptome libraries were used for differential expression analysis. The *in vitro* transcript analysis was performed using 27 tissue-specific transcriptome libraries from MPOB that were submitted to GenBank under BioProject PRJNA201497 and PRJNA345530 (Singh *et al.*, 2013), four root libraries from BioProject PRJEB7252 (Ho *et al.*, 2016), 51 libraries from mesocarp at different development stages (Morris *et al.*, 2020) (In-house project B), 28 libraries from different kernel development stages (In-house project B) (unpublished data) and 34 transcriptome libraries of roots (In-house project A). In-house project A contains datasets of roots that were infected with *G. boninense*, *G. boninense* and mycorrhiza, and controls (no infection) (unpublished data). In-house project B contains datasets from mesocarp at 5, 8, 10, 12, 15, 18, 20, 22 and 24 weeks after anthesis (WAA), and kernel at 8, 10, 12, 15 and 18 WAA. All transcriptome libraries were sequenced using Illumina sequencing technique except PRJNA201497 and PRJNA345530, which were generated using Roche/454 GS FLX Titanium (Roche/454) sequencing platform (*Table 1*).

The sequencing raw reads were trimmed using Trim Galore version 0.4.0 with Phred score >20 and length >30 bp. Read-mapping and expression analysis was performed using Tuxedo suite pipeline (Trapnell *et al.*, 2009). The reads from each library were mapped to *E. guineensis* P5-genome build (Singh *et al.*, 2013) using Tophat 2.0.9 with an intron length of 30 bases to 50 kb, followed by assembly using Cufflinks 2.2.1 with default parameters (Trapnell *et al.*, 2010). The assemblies of all the libraries were then merged using Cuffmerge 2.2.1, and the expression data were processed by Cuffdiff 2.2.1. Geometric fragment per kilobase per million mapped reads (FPKM) was calculated in Cuffdiff 2.2.1 to normalise the transcript expression levels. Systematic mining of the transcriptome data to select candidates for the root-specific promoter was carried out using Microsoft Excel. Transcripts that were smaller than 300 bp in length were removed from further analysis.

Isolation of Total RNA

Total RNA was extracted from 24 different oil palm tissues, including root tissues collected from 12 month-old tissue culture plantlets (RS1), 16 month-old plantlets that were planted in jiffy pots in the nursery (RS2), primary and lateral roots from 24 month-old oil palm seedlings (RS3), primary and lateral roots of 10 year-old oil palm (RS4), male inflorescences, female inflorescences, green leaves, young leaves, cabbage, callus, polyembryoids, plantlets (at tissue culture stage and not rooting yet), mesocarps and kernels. Isolation of total RNA of oil palm tissues was carried out using the method described by Zeng and Yang (2002). This is a simple method with modifications of the cetyltrimethyl ammonium bromide (CTAB) buffer and soluble polyvinylpyrrolidone (PVP). The utilisation of CTAB was suitable for RNA isolation of oil palm that contains a high level of phenolic compounds. The addition of PVP into the extraction buffer releases the RNA from lipids as the PVP forms complexes with polysaccharide and polyphenol compounds. The total RNA was dissolved in the

TABLE 1. OIL PALM TRANSCRIPTOME DATA

RNA-Seq Project	Number of trancriptome libraries	Sequencing platform	Layout	Number of reads
In-house project A	34	Illumina	paired-end	28.0-77.0M
In-house project B	79	Illumina	paired-end	15.0-89.0M
BioProject PRJEB7252	4	Illumina	1 paired + 3 single	16.0-19.0M
BioProject PRJNA201497	22	Roche/454	single-end	0.3-0.6M
BioProject PRJNA345530	5	Roche/454	single-end	1.1-1.3M

nuclease-free water and stored at –80ºC. Treatment of total RNA with RNase-free DNase and RNeasy Mini Kit (Qiagen USA, Valencia, CA) was carried out to remove deoxyribonucleic acid (DNA) contamination. NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc.) was used to quantify the RNA quantity and purity. Simultaneously, the RNA integrity and quality were assessed through the RNA Quality Number (RQN) using Fragment Analyser™ (Advanced Analytical Technologies, Inc.).

Real Time-quantitative PCR (RT-qPCR)

First-strand complementary DNA (cDNA) was synthesised using the High-capacity cDNA Reverse-Transcription Kit following the instruction described by the manufacturer (Applied Biosystems). Reverse transcription was carried out using 2 µg of total RNA, which yielded about 100 ng of cDNAs. PCR amplification efficiencies and correlation coefficient (R^2) of each primer pair were calculated using a standard curve generated using a two-fold serial dilution of the pooled cDNAs from roots (2, 4, 6, 8, 16 and 32 ng). The RT-qPCR based on SYBR Green was carried out using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad) in 96-well plates. About 16 ng of cDNA was used in a 20 µL quantitative reaction mix containing 1x iTaq Universal SYBR Green Supermix (2X), 0.5 µM of forward primer and 0.5 µM of reverse primer. The protocol for RT-qPCR is as followed; 95.0°C, 30 min for one cycle and 30 s at 95.0°C and 30 s at 45.0°C (depending on the optimal primer annealing temperature) for 40 cycles. Each sample was analysed in three technical replicates (n=3). The melting curve for each amplicon was obtained from 65.0°C-95.0°C with a 0.5°C increase in temperature at each step. The relative fold difference of expression for each sample in each experiment was determined by normalising the mean cycle quantification (Cq) value for each gene to the mean Cq value of reference genes (Gibberellin-responsive protein 2 (*GRAS*), pre-messenger ribonucleuc acid (mRNA) splicing factor 7 (*SLU7)* and *PD00569*, and calculated relative to a calibrator using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). The expression profiles of the transcripts were analysed using BioRad CFX Manager™ 3.0 software (BioRad). Significance fold change of expression for each gene was measured using the Student t-test with $p<0.01$.

Sequence Analysis

Nucleotide sequences were annotated using GenBank Plant Reference Sequence (RefSeq) Database (OLeary *et al.*, 2016) via BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1997) with default parameters. Functional gene annotation was performed by searching the amino acid or protein sequence homology in a non-redundant RefSeq protein database (Pruitt *et al.*, 2005) by using BLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi) alignments with an e-value threshold of 1^{e-5}. Nucleotide and amino acid sequences of the targeted gene and its counterpart from other plants that were retrieved from GenBank were deposited to Vector NTI® software (Thermo Fisher Scientific Inc.) (Lu and Moriyama, 2004). The open reading frame of the targeted gene was translated to the corresponding amino acid sequence by using a translation tool in the software. Then, multiple alignments of nucleotides or amino acid sequences of the targeted gene and its counterpart were performed using AlignX tool based on the ClustalW algorithm.

RESULTS AND DISCUSSION

Identification of Putative Root-specific Genes via RNA-Seq Analysis

RNA-Seq data from the root, shoot, fruit, inflorescence, pollen, leaf, mesocarp, kernel, pith, sepal, spikelet, and stalk were used to screen for candidates for root-specific or preferentially expressed genes. The transcripts were mapped to MPOB's *AVROS Pisifera* genome P5-build. A total of 51 889 genes with 165 751 isoforms were obtained from the RNA-Seq analysis. Transcripts smaller than 300 bp in length were removed, as these sequences could result in slight over-estimation of the expression abundance, which could lead to misinterpretation in the data analysis. The short reads could have arisen from incomplete contig assembly (Hsieh *et al.*, 2019) or belong to the small or non-coding RNA (Liu *et al.*, 2019). The analysis resulted in the discovery of genes that were predominantly but not specifically expressed in the root. Among the 159 490 filtered transcripts, seven transcripts (TCONS_00011027, TCONS_00000859, TCONS_00140324,TCONS_00083022,TCONS_00044801, TCONS_00110826, and TCONS_00034877) were found to be the most highly expressed in root tissues. These transcripts only had low levels of expression detected in other tissues (*Figure 1*) and were therefore chosen for further analysis. It is noteworthy from the findings of Evans *et al.* (1988) that showed that the efforts in isolating root-specific cDNA clones from pea were unsuccessful and that they concluded that root-specific mRNA species (if present) were only present at very low levels of abundance in root mRNA populations. Choosing a transcript that has low levels of expression may not be suitable for the identification of promoters to regulate transgene expression, as promoters that possess strong activity are desired for this purpose.

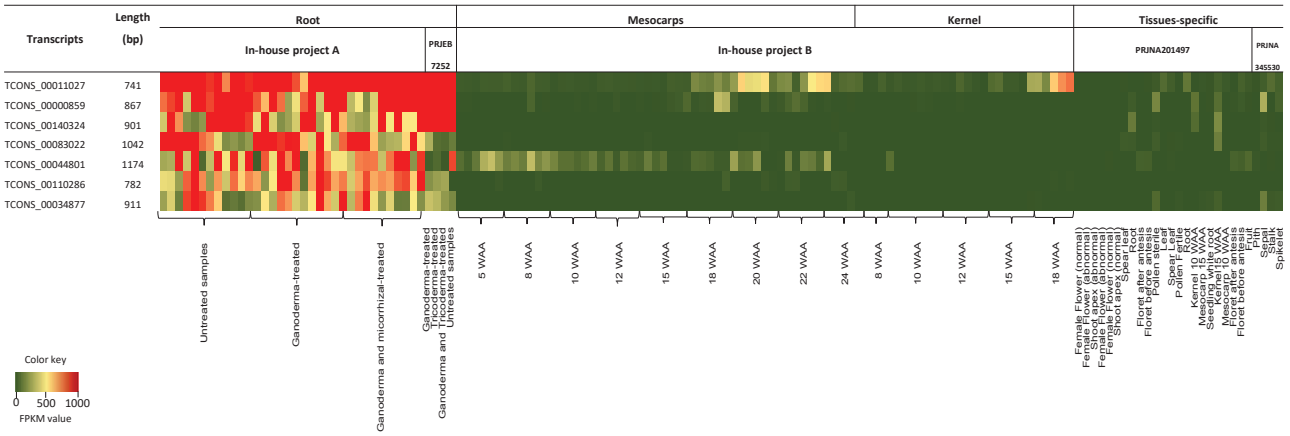

Figure 1. Mining of oil palm transcriptome data using the RNA-Seq approach. A total of seven candidate root-specific or root preferential expressed genes were selected from the in silico *analysis. Transcriptome libraries were derived from GenBank BioProject PRJNA201497, PRJNA345530, PRJEB7252, root (In-house project A), and mesocarp and kernel from In-house project B. Red and green shades indicate higher and lower expression, respectively. The colour key indicates the intensity associated with normalised expression value using the FPKM method.*

High-quality RNA for RT-qPCR

The expression pattern of the genes selected through RNA-Seq analysis was further validated using RT-qPCR analysis. To perform RT-qPCR, the isolation of high-quality total RNA is essential. In this study, total RNA was extracted from 24 different oil palm tissues including root tissues at different developmental stages (RS1, RS2, RS3 and RS4), male inflorescence, female inflorescence, green leaves, young leaves, cabbage, callus, polyembryoids, mesocarp and kernel. About 3.84-31.35 µg g^{-1} fresh weight tissue of total RNA was obtained. The total RNA was of high purity, as the A_{260}/A_{280} and A_{230}/A_{260} ratios for all samples were greater than 1.8, indicating the absence of protein and other organic compounds (Claros and Canovas, 1999). RNA integrity and quality were also good, as the RQN values were relatively high, ranging from 7 to 9 (*Table 2*).

Tissue Specificity Analysis through RT-qPCR Analysis

A standard curve of PCR amplification efficiency and R^2 was generated for each reference and target gene. The amplification efficiency value for all reference and target genes tested was within the range of 90% and 110%, while the R^2 value was >0.98, indicating a positive correlation between the amount of cDNA template and the cycle threshold (Ct) values (Bustin *et al.*, 2009). *Table 3* shows the primers used for target genes and the value of amplification efficiency and R^2 of the standard curve generated for RT-qPCR. The temporal and spatial expressions of targeted genes were evaluated across the 24 different oil palm tissues. The quantitative data for gene expression was normalised to the expression level of reference genes, namely *GRAS*, *SLU7* and *PD00569*, that have been documented as stable reference genes in oil palm (Chan *et al.*, 2014; Yeap *et al.*, 2014). The root tissue at the early development stage (RS1) was used as a calibrator. The selection of RS1 as a calibrator will give a better understanding of transcripts expression patterns in roots. The expression profiles of the genes can be measured from the earlier to the later stages of root development, while a significant comparison to other tissues (non-root) will give a good indication of their specificity.

Expression analysis of the putative root-specific genes using RT-qPCR is shown in *Figure 2*. The relative fold change of expression was measured in $\log_2$ ratio and statistically validated using t-test with $p<0.01$. The results showed that TCONS_00011027 was highly expressed in RS1 as the average Cq value was detected at 16. The transcript was highly expressed at the early stages of root development as no fold change in expression was observed in RS1, RS2 and lateral roots of RS3. Although the gene was significantly downregulated (~6 to 42-fold) at the later stages of root development [RS3 (primary root) and RS4], its expression was still relatively high, as the RT-qPCR amplification plots showed average Cq values at 16 to 22 cycles. Likewise, the transcript also showed a significant downregulation in the non-root tissues. However, the transcript's expression level in callus, polyembryoids, young and green leaves overlaps the expression in roots, indicates that it is not a root-specific or root-preferentially expressed gene.

TABLE 2. YIELD AND PURITY OF TOTAL RNA ISOLATED FROM VARIOUS OIL PALM TISSUES

Sample	Yield (μg g^{-1})	A260/A280	A260/A230	RNA quality number (RQN)
C	6.29	2.16	2.13	8.8
CB	19.90	2.06	2.01	8.9
FI	10.27	2.08	1.98	7.8
MI	9.19	2.10	1.95	8.2
GL	15.78	2.14	2.00	8.4
YL	13.92	2.19	2.18	9.0
K15	10.22	2.08	1.80	8.8
M15	19.20	2.06	1.96	9.0
PE	6.10	2.10	1.87	8.6
PL	19.60	2.07	2.11	9.0
RS1	3.84	2.05	1.80	7.6
RS2	4.78	2.05	2.10	7.0
RS3 (LR1)	11.40	2.15	2.27	7.9
RS3 (LR2)	28.58	2.08	1.83	7.6
RS3 (LR3)	30.73	2.06	2.03	7.7
RS3 (PR1)	21.08	2.11	2.27	8.8
RS3 (PR2)	31.35	2.02	2.18	9.0
RS3 (PR3)	25.95	2.10	2.23	8.6
RS4 (LR1)	15.80	2.14	1.81	8.4
RS4 (LR2)	24.22	2.09	2.29	7.4
RS4 (LR3)	31.06	2.02	2.21	8.6
RS4 (PR1)	24.47	2.10	2.28	7.4
RS4 (PR2)	12.51	2.15	2.25	7.0
RS4 (PR3)	14.70	2.14	1.80	7.4

Note: C - callus; CB - cabbage; FI - female inflorescence; MI - male inflorescence; GL - green leaves; YL - young leaves; K15 - kernel 15 WAA; M15 - mesocarp 15 WAA; PE - polyembryoids; PL - plantlets; RS1 - root from plantlets (12 months); RS2 - root planted in jiffy pot (16 months); RS3 (LR1), RS3 (LR2), RS3 (LR3), RS3 (PR1), RS3 (PR2) and RS3 (PR3) - lateral and primary roots in polybag (24 months); and RS4 (LR1), RS4 (LR2) and RS4 (LR3), RS4 (PR1), RS4 (PR2) and RS4 (PR3) - lateral and primary roots at field planting (10 years).

TABLE 3. PRIMERS OF PUTATIVE ROOT SPECIFIC GENES FOR RT-qPCR

Targeted gene	Primer sequence (5′-3′)	Amplification length (bp)	Annealing temperature (°C)	Amplification efficiency (%)	Regression coefficient (R^2)
TCONS_00011027	F-TTGGTTGTTGTAGTTCTTCATATTAG R-GGTGCTGGTCTTCTCAGCCA	140	48	96.8	0.992
TCONS_00000859	F-GCTTCGGCATTGTGACACT R-GCAGTTGGAGCTGCACTTGC	101	50	93.7	0.984
TCONS_00140324	F-TGGAAAATGGCTTCCAAGTC R-GATCCAGAAGAAGGTGAGGG	146	48	97.1	0.980
TCONS_00083022	F-GCCTAGGAAACAATCAAGTTTAACG R-ATACCGGCGGCGCTTGCGATGACATT	155	45	105.4	0.993
TCONS_00044801	F-CACAAATTTCAGACAAGCAGC R-CAGACTTTCTCACAGACACAGAACA	156	47	90.9	0.983
TCONS_00110826	F-AAGATGAGACGCCACAA R-CAGACTTTCTCACAGACACAGAACA	149	51	92.1	0.993
TCONS_00034877	F-CCGGACACAAACCACCAACT R-GGCTTCTCATACGGTTTGGG	139	45	91.1	0.989

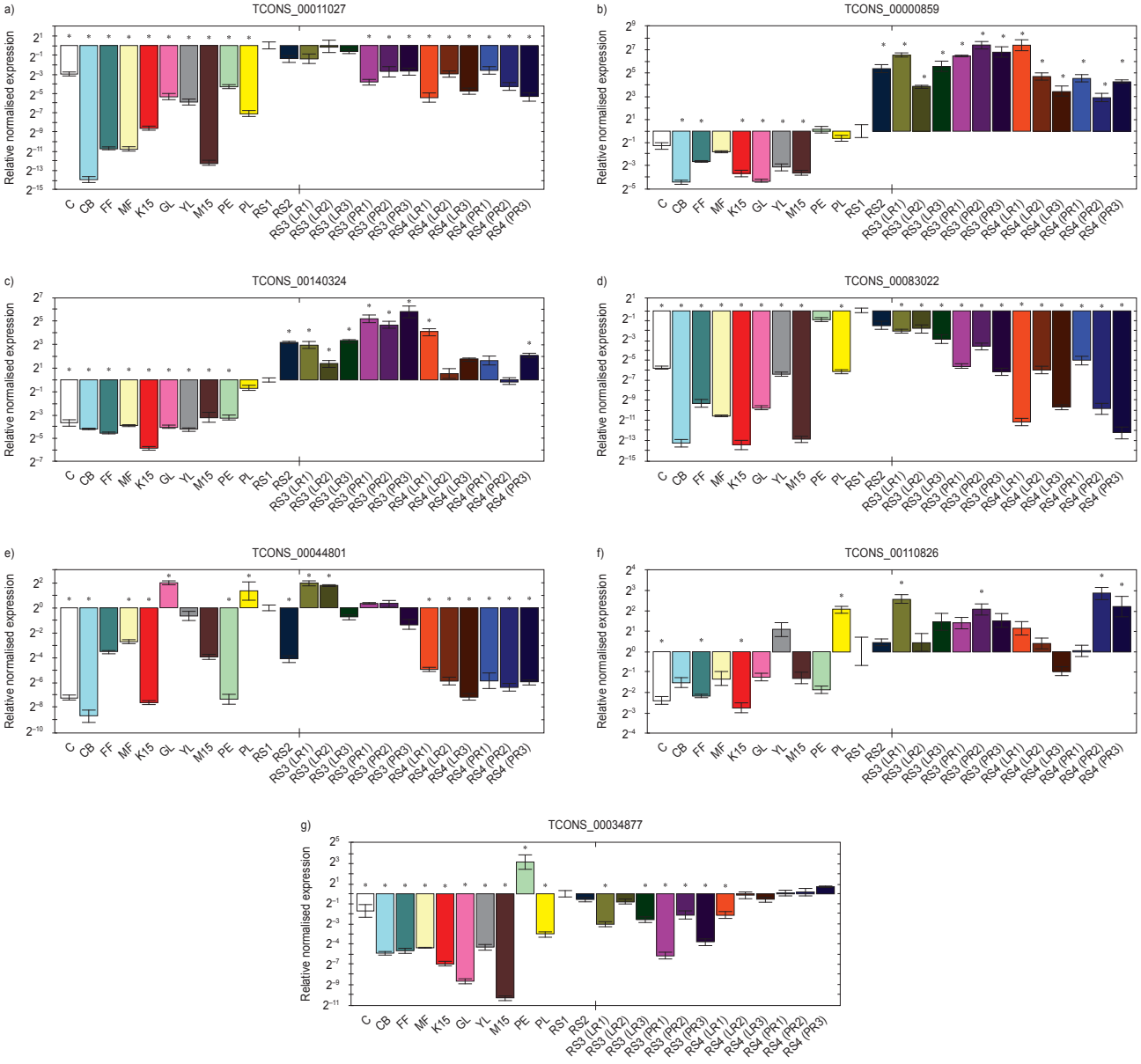

Figure 2. Expression analysis of putative root-specific genes using RT-qPCR. Graphs indicate the expression data of a) TCONS_00011027, b) TCONS_00000859, c) TCONS_00140324, d) TCONS_00083022, e) TCONS_ 00044801, f) TCONS_00110826, and g) TCONS_00034877. Y-axis shows the average relative expression of the transcripts calculated using $2^{-\Delta\Delta Cq}$ against reference genes. Expression levels of the transcripts were compared with RS1 as a calibrator. The average quantification cycle (Cq) value of RS1 for TCONS_00011027, TCONS_00000859, TCONS_00140324, TCONS_00083022, TCONS_00044801, TCONS_00110826, and TCONS_00034877 were 16, 27, 24, 19, 20, 27 and 16, respectively. Three technical replicates were used for each biological sample. Three biological samples were used for primary and lateral roots in polybag (24 months) [RS3 (PR1), RS3 (PR2), RS3 (PR3), RS3 (LR1), RS3 (LR2) and RS3 (LR3)] and primary and lateral roots at field planting (10 years) [RS4 (PR1), RS4 (PR2), RS4 (PR3), RS4 (LR1), RS4 (LR2) and RS4 (LR3)] and one biological sample used for root from plantlets (12 months) (RS1) and nursery stage (planted in jiffy pot (16 months) (RS2), callus (C), male inflorescence (MI), female inflorescence (FI), green leaves (GL), young leaves (YL), cabbage (CB), polyembryoids (PE), plantlets (PL), mesocarp at 15 WAA (M15) and kernel at 15WAA (K15). Error bars indicate the standard error of the mean of three technical replicates. Asterisks indicate the significance fold change of transcript expression compared to RS1 as a calibrator (t-test: $p<0.01$).

On the other hand, TCONS_00000859 had a low expression level in RS1, as the RT-qPCR amplification plots showed average Cq values at 27. However, the gene expression was markedly increased across the different developmental stages of root with a significant upregulation of around 7 to 170-fold. It was notable that the expression of the transcript in non-root tissues was relatively low compared to most of the root tissues. All the non-root tissues had either no significant fold change in comparison to RS1 (callus, male inflorescence, polyembryoids and plantlets) or was significantly downregulated by 6 to 21-fold (cabbage, female inflorescence, kernel, leaves, and mesocarp). A similar gene expression pattern was seen for TCONS_00140324, which had a moderate

level of expression in RS1 (average Cq value of 24). In addition to a comparable expression of TCONS_00140324 in the later stages of root development [RS4(LR2), RS4(LR3), RS4(PR1) and RS4(PR2)], a significant upregulation of the transcript at around 7 to 55-fold was observed across other developmental stages of the root. Apart from plantlets that showed no differential expression with RS1, the transcript was downregulated in callus, polyembryoids, cabbage, inflorescence, kernel, mesocarp and leaves by about 9 to 57-fold. These results were generally in concordance with RNA-Seq data that showed that TCONS_00140324 and TCONS_00000859 transcripts were observed in abundance in roots, with low or barely detectable expression in other tissues.

Over and above, we found that the TCONS_00083022 and TCONS_00044801 transcripts were highly expressed in RS1 as an average amplification plot of Cq was detected at 19 and 20 cycles, respectively. However, TCONS_00083022 was significantly downregulated across all tissues tested, except in polyembryoids that showed no significant fold change in expression. For TCONS_00044801, the transcript was only upregulated in green leaves, plantlets and RS3 (LR1 and LR2) by about 4-fold while significantly downregulated in other tissues, including RS2 and RS4. Overall, the expression levels of these two transcripts in root tissues overlap with the expression levels in the non-root tissues, indicating that the genes are not preferentially expressed in root tissues.

For TCONS_00110826, a noticeable low expression of the transcript was observed in RS1 with an average Cq value of 27. The transcript was significantly upregulated in root tissues of RS3 (LR1 and PR2), RS4 (PR2 and PR3) and plantlets at around 4 to 6-fold, while significant downregulation was observed in callus, female inflorescence and kernel. No significant fold change in expressions was observed in the other tissues studied, indicating that the gene expression was constant but relatively low, even in the root development samples. For TCONS_00034877, the gene was expressed in RS1 with an average Cq value of 16. Although the RT-qPCR profiles in root had indicated either downregulation of around 4 to 33-fold or no significant fold change in expression, the expression levels of the gene in root tissues overlaps with the majority of the non-root tissues. The gene seems to be constitutively expressed, suggesting a possible housekeeping role in oil palm tissues.

Although the RNA-Seq and RT-qPCR data were generally in agreement, particularly for expression profiles of TCONS_00000859 and TCONS_00140324, some results differed. Discrepancies between the RNA-Seq and RT-qPCR data were observed, particularly for TCONS_00083022, TCONS_00044801, TCONS_00110826, and TCONS_00034877. The differences could be attributed to the different biological samples or materials used in both platforms. The background of the biological samples used for the RNA-Seq experiments varies, as the samples came from *Tenera, Dura* or *Pisifera* palms. In contrast, the RT-qPCR experiments were conducted using tissue culture-derived ramets of P456 clone (*Tenera* palm) (Zulkifli *et al.*, 2017). The variability of biological materials derived from different genetic backgrounds might contribute to the variation in expression quantification.

Further validation of the transcript expression profiles through RT-qPCR is essential and fundamental as this method is highly sensitive for gene quantification and can be highly sequence-specific (Costa *et al.*, 2013). To further validate and obtain an accurate result, the RT-qPCR was conducted using biological materials derived from the same genetic background with three technical replicates for each sample. This has increased RT-qPCR precision, improved experimental variation, and served to improve confidence as a better estimation of the mean is provided by the technique (Sanders *et al.*, 2014).

Selection of Putative Root-specific Genes

Based on spatial and temporal expression measured by RT-qPCR, the high abundance of TCONS_00011027 and TCONS_00034877 transcripts were not only observed in the root but also the other tissues studied. Based on the annotation to the non-redundant RefSeq protein database in Genbank, TCONS_00011027 and TCONS_00034877 were similar to metallothionine (XM_010924034.3) and early nodulin-75-like genes (ENOD2) (XM_010942463.1), respectively. In legume, the early nodulin-75-like ortholog was involved in the *early* stages of root nodule development (Franssen *et al.*, 1987). The gene codes for a proline-rich protein, which is a part of the structural protein component of the plant cell wall. Strong expression of the gene in oil palm may coincide with its role as structural wall protein involved in important developmental processes, such as vascular differentiation, wound healing, or defence response against pathogens (Wilson *et al.*, 1994). However, it is notable that early nodulin-like proteins belong to a multigene family. Although nodulin genes had already been cloned and sequenced, the functions for many of them are sparsely described (Tikhonovich and Provorov, 2007).

For TCONS_00083022, the gene showed higher accumulation in tissue culture samples (callus, polyembryoids and RS1) and the earlier stages of root development (RS2 and RS3). While lower

levels of expression were detected in RS4 and the other tissues studied. The gene is similar to pathogenesis-related protein 1 (*PR-1*) (Genbank accession no: XM_010940037.3) that belongs to a multigene family. Pathogenesis-related proteins (PR) are a structurally diverse group of plant proteins that are toxic to invading fungal pathogens (Agrios, 2005). PR-1 protein has been reported to have antimicrobial activity.

In addition to the pathogen attack response, *PR-1* genes are also responsive to abiotic stimuli, suggesting their important roles in abiotic stress response (Breen *et al.*, 2017). The elevated expression of TCONS_00083022 in tissue culture samples (callus, polyembryoids) RS1, RS2 and RS3 may be associated with abiotic stimuli. Controlled stress in *in vitro* cultures, such as mechanical injuries, oxidative stress, and high plant growth regulator concentrations, may stimulate stress-related genes, such as *PR-1*. At the RS2 and RS3 stages, oil palm seedlings in polybags may also encounter growth stresses, such as water deficit, dry heat and low humidity that could interfere with the root elongation process. In contrast to RS4, which is planted in peat soil, the high-water table and loose soil structure of peat soil may have made it easier for root growth and elongation, likely reducing the induction of stress-related genes. Interestingly, as the gene shows expression patterns related to plant defence response, its promoter may be inducible, making it useful to fine-tune the expression of transgenes in response to pathogen attack or abiotic stresses. On the other hand, TCONS_00044801 showed varying expression levels at different fold changes across all the tissues tested, while TCONS_00110826 showed relatively low levels of expression. The roles and regulation of these genes are yet to be understood as they are classified as proteins of unknown function.

This study showed that TCONS_00000859 and TCONS_00140324 transcripts were highly expressed in the root and barely detected in other tissues. As the results demonstrate the potential of their promoters to drive strong expression of transgenes in a root-specific manner, the sequences of TCONS_00000859 and TCONS_00140324 were analysed in detail. The first gene, named *EgMT* codes for the TCONS_00000859 transcript, had significant similarity to an oil palm metallothionein sequence (Genbank accession no: MK557924.1). The gene belongs to the Class II metallothionein (MT) gene family. *EgMT* has a 192 bp open reading frame that encodes a 63 amino acid polypeptide with a theoretical molecular mass of 6.58 kDa and a pI of 4.65. Multiple amino acid alignments conducted on *EgMT* and its counterparts from other plants such as from *Asparagus officinalis* (XP_020267036.1), *Fritillaria agrestis* (AAB95221.1), *Dracaena cambodiana* (ASR83111.1), *Ananus comosus* (OAY84410.1) and *Metroxylon sagu* (ABA43635.1), as shown in *Figure 3*, indicates that sequence similarity was around 64.06%-70.77%. A detailed comparison analysis was also carried out between *EgMT* and *MT3-B* sequences (another oil palm root-specific promoter). Using a pairwise sequence alignment tool (https://www.ebi.ac.uk/Tools/psa/emboss_needle/), results showed that these genes share about 89.60% similarity in their coding regions and 21.70% similarity in both their 5′ and 3′ non-coding regions. This data indicates that *EgMT* belongs to another family of oil palm MT genes. Based on nucleotide search to the National Center for Biotechnology Information (NCBI), oil palm has at least five MT genes that belong to different types of MT (data not shown). The detailed analysis of *EgMT* indicated that it contains the C-X-C motif, which is essential for effective metal binding. This cysteine-rich metal-binding protein is vital as MTs are involved in various cellular functions such as protection against oxidative stress, zinc and copper homeostasis, and buffering against toxic heavy metals (Joshi *et al.*, 2016; Shabb *et al.*, 2017).

Determination of spatial and temporal expression of *EgMT* through RT-qPCR indicated that *EgMT* is one of the MT types that are preferentially expressed in the root. The expression of *EgMT* was in contrast to a metallothionine coded by TCONS_00011027 that showed high abundance in root and other tissues including callus, polyembryoids, young and green leaves. Cobbett and Goldsbrough (2002) reported that MTs of higher plants were classified into several types (Foley and Singh, 1994; Guo *et al.*, 2003; Reid and Ross, 1997). The diverse expression patterns of different MT genes suggest that plant MT isoforms may differ in sequence and in the functions they perform in specific tissues (Cobbett and Goldsbrough, 2002). Although many genes encoding MTs have been isolated and characterised, their precise functions and regulation are not entirely understood (García-Hernández *et al.*, 1998). Interestingly, in addition to oil palm *MT3-B* that has been documented as an inducible root promoter, Dong *et al.* (2010) also reported *OsMT-I-4b* as an inducible root promoter from rice. The potential of *EgMT* promoter to regulate transgenes in a root-specific manner is evident by the abundance of its transcript in root based on RT-qPCR and RNA-Seq. However, further characterisation of the promoter via deletion analysis may reveal the critical regulatory regions of *EgMT* that are essential for root-specific regulation.

TCONS_00140324, coded by a gene named *EgPRP1*, is similar to a 14kD proline-rich protein (Genbank accession no: XM_010908304.3). In general, proline-rich proteins belong to the hybrid proline- or glycine-rich protein (HyP/GRP) gene family that functions as plant-specific and putative

cell-wall/plasma membrane-associated proteins (Fujino *et al.*, 2014). The EgPRP1 protein consists of 132 amino acids, including 14 (11.45%) proline residues, with a theoretical molecular mass of 13.44 kDa and a pI of 9.27. The protein also consists of a hydrophobic domain at the N terminus that represents a signal sequence. The EgPRP1 protein sequence is most similar (63.91%-93.02% identical) to a set of putative cell wall-localised proline-rich proteins isolated from several plant species, such as *Phoenix dactylifera* (XP_008808738.1), *A. comosus* (XP_020085092.1), *Glycine max* (XP_003525817.1), *Citrus sinensis* (XM_006477171.2) and *Sesamum indicum* (XP_011081229.1) (*Figure 4*).

Figure 3. Multiple sequence alignment of EgMT *with various homologous sequences retrieved by BLASTx analysis. The* EgMT *gene showed homology to metallothionein genes from* Asparagus officinalis *(XP_020267036.1)[AoMT],* Fritillaria agrestis *(AAB95221.1) [FaMT],* Dracaena cambodiana *(ASR83111.1)[DcMT],* Ananus comosus *(OAY84410.1) [AcMT] and* Metroxylon sagu *(ABA43635.1)[MsMT] with sequence similarity of around 64.06%-70.77%. Multiple sequence alignment showed conserved C-X-C motifs (highlighted in box). Gaps introduced for best alignment are shown as dash.*

Figure 4. Multiple sequence alignment of EgPRP1 *with various homologous sequences retrieved by BLASTx analysis. The* EgPRP1 *gene showed homology to proline-rich protein genes from* Ananas comosus *(XP_020085092.1)[AcPRP],* Phoenix dactylifera *(XP_008808738.1)[PdPRP],* Glycine max *(XP_003525817.1)[GmPRP],* Citrus sinensis *(XM_006477171.2) [CsPRP]) and* Sesamum indicum *(XP_011081229.1)[SePRP], with sequence similarity of around 63.91%-93.02%. Gaps introduced for best alignment are shown as dash.*

A similar expression pattern as *EgMT* was observed in the RT-qPCR results. High levels of expression of *EgPRP1* were markedly detected in roots. The high activity *of EgPRP1* in roots may correlate to the PRP function involved in cell and root elongation. Several studies have described the discovery of PRP genes with specific or preferential expression in root. For examples, the specific expression of *Nicotiana tobaccum* and soybean hydroxyproline-rich glycoprotein in the endodermal cells of the zone that lateral roots emerge, are necessary to provide the mechanical strength required for penetrating through the main root, as the genes are considered to be involved in cell wall reformation (Ahn *et al.,* 1996) and hardening (Keller and Lamb, 1989). Several studies also reported that rice *RCc2* and *RCc3*, alfalfa *A9*, maize *ZRP3*, carrot *PRP1*, bean *PVR5*, *Vitis vinifera PRP2*, *Medicago sativa PRP2*, soybean *PRP1* and *PRP2* genes, which all encode a proline-rich protein, are expressed preferentially in the root (Choi *et al.,* 1996; Ebener *et al.,* 1993; Jeong *et al.,* 2010; Winicov *et al.,* 2004; Xu *et al.,* 1995). This indicates that the findings of *EgPRP1* are in agreement with these previous studies. On the other hand, a unique expression of *EgPRP1* has also been detected in the plantlets. The long duration of plantlet cultivation in tissue culture media triggered various stresses that may lead to the expression of *EgPRP1*. The result is in accordance with previous researches that showed abiotic and biotic stresses influenced the expression of the PRP genes (Bhattacharya *et al.,* 2013; Srinath *et al.,* 2017; Suzuki *et al.,* 1993). Nonetheless, the comprehensive studies of PRPs have revealed that PRPs act in various plant growth processes to meet the functional and physical requirements of different cell types at different developmental stages (Josè-Estanyol *et al.,* 1992; Showalter and Rumeau, 1990). For example, in addition to the exclusive expression of *AtPRP1* and *AtPRP3* in roots, other *Arabidopsis* PRPs, namely *PRP2* and *PRP4* transcripts, were abundant in the plant's aerial organs (Bernhardt and Tierney, 2000; Fowler *et al.,* 1999). PRPs' promoter's ability in driving the gene of interest or reporter genes has also been studied. Several reports documented that *RCc3, AtPRP1, AtPRP3, MsPRP2* and *GmPRP2* can drive the expression of transgenes in a root-specific manner (Chen *et al.,* 2014; Fowler *et al.,* 1999; Jeong *et al.,* 2010; Li *et al.,* 2019; Winicov *et al.,* 2004).

CONCLUSION

The transcript profiling studies through RNA-Seq have accelerated the discovery of novel root-specific or preferential genes for promoter isolation. RNA-Seq data analysis identified seven candidate genes that potentially have root-specific or preferential expression in roots. Validation experiments showed that in general, the RNA-Seq expression profiles are correlated well with the RT-qPCR data. Some of the discrepancies observed were possibly due to the different biological materials used in both transcript profiling platforms. Out of the seven selected candidates, we found two novel oil palm transcripts, *EgMT* and *EgPRP1*, that were highly expressed in the root and barely detectable in other tissues. The results greatly suggest the potential of *EgMT* and *EgPRP1* promoters in regulating the strong expression of transgenes in a root-specific manner.

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REFERENCES

Agrios, G N (2005). Chapter 6 - How plants defend themselves against pathogens. *Plant Pathology* (Agrios, G N ed.) 5th edition. Academic Press, San Diego. p. 207-248.

Ahmadi, F; Akmar Abdullah, S N; Kadkhodaei, S; Ijab, S M; Hamzah, L; Aziz, M A; Rahman, Z A and Rabiah Syed Alwee, S S (2018). Functional characterization of the gene promoter for an *Elaeis guineensis* phosphate starvation-inducible, high affinity phosphate transporter in both homologous and heterologous model systems. *Plant Physiol. Biochem., 127*: 320-335.

Ahn, J H; Choi, Y; Kwon, Y M; Kim, S-G; Choi, Y D and Lee, J S (1996). A novel extensin gene encoding a hydroxyproline-rich glycoprotein requires sucrose for its wound-inducible expression in transgenic plants. *Plant Cell, 8*: 1477-1490.

Alpern, D; Gardeux, V; Russeil, J; Mangeat, B; Meireles-Filho, A C A; Breysse, R; Hacker, D and Deplancke, B (2019). BRB-seq: Ultra-affordable high-throughput transcriptomics enabled by bulk RNA barcoding and sequencing. *Genome Biol., 20(1)*: 71. DOI: 10.1186/s13059-019-1671-x.

Altschul, S F; Madden, T L; Schäffer, A A; Zhang, J; Zhang, Z; Miller, W and Lipman, D J (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res., 25(17)*: 3389-3402. DOI: 10.1093/nar/25.17.3389.

Bernhardt, C and Tierney, M L (2000). Expression of AtPRP3, a proline-rich structural cell wall protein from *Arabidopsis,* is regulated by cell-type-specific developmental pathways involved in root hair formation. *Plant Physiol., 122*: 705-714.

Bhattacharya, R; Koramutla, M K; Negi, M; Pearce, G and Ryan, C A (2013). Hydroxyproline-rich glycopeptide signals in potato elicit signalling associated with defense against insects and pathogens. *Plant Sci., 207*: 88-97.

Breen, S; Williams, S J; Outram, M; Kobe, B and Solomon, P S (2017). Emerging insights into the functions of pathogenesis related protein 1. *Trends Plant Sci., 22(10)*: 871-879. DOI: 10.1016/j.tplants.2017.06.013.

Bustin, S A; Benes, V; Garson, J A; Hellemans, J; Huggett, J; Kubista, M; Mueller, R; Nolan, T; Pfaffl, M W; Shipley, G L; Vandesompele, J and Wittwer, C T (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem., 55(4)*: 611-622.

Chan, P L; Rose, R J; Abdul Murad, A M; Zainal, Z; Leslie Low, E T; Li Ooi, L C; Eng Ooi, S; Suzaini, Y and Singh, R (2014). Evaluation of reference genes for quantitative real-time PCR in oil palm elite planting materials propagated by tissue culture. *PLoS ONE, 9(6)*: e99774. DOI: 10.1371/journal.pone.0099774.

Chen, L; Jiang, B; Wu, C; Sun, S; Hou, W and Han, T (2014). *GmPRP2* promoter drives root-preferential expression in transgenic *Arabidopsis* and soybean hairy roots. *BMC Plant Biol., 14*: 245.

Choi, D W; Song, J Y; Kwon, Y M and Kim, S G (1996). Characterization of cDNA encoding a proline-rich 14 kDa protein in developing cortical cells of the roots of bean (*Phaseolus vulgaris*) seedlings. *Plant Mol. Biol., 30*: 973-982.

Claros, M G and Canovas, F M (1999). RNA isolation from plant tissues: A practical experience for biological undergraduate. *Biochem. Educ., 27*: 110-113.

Cobbett, C and Goldbrough, P B (2002). Phytochelatins and metallothionein: Roles in heavy metal detoxification and homeostasis. *Annu. Rev. Plant Biol., 53*: 159-182.

Costa, C; Giménez-Capitán, A; Karachaliou, N and Rosell, R (2013). Comprehensive molecular screening: From the RT-PCR to the RNA-seq. *Transl. Lung Cancer Res., 2(2)*: 87-91. DOI: 10.3978/j.issn.2218-6751.2013.02.05.

Dong, C J; Wang, Y; Yu, S S and Liu, J Y (2010). Characterization of a novel rice metallothionein gene promoter: Its tissue specificity and heavy metal responsiveness. *J. Integr. Plant Biol., 52(10)*: 914-924. DOI: 910.1111/j.1744-7909.2010. 00966.x.

Ebener, W; Fowler, T J; Suzuki, H; Shaver, J and Tierney, M L (1993). Expression of DcPRP1 is linked to carrot storage root formation and is induced by wounding and auxin treatment. *Plant Physiol., 101*: 259-265.

Evans, I M; Swinhoe, R; Gatehouse, L N; Gatehouse, J A and Boulter, D (1988). Distribution of root mRNA species in other vegetative organs of pea (*Pisum sativum* L.). *Mol. Gen. Genet., 214(1)*: 153-157. DOI: 10.1007/BF00340194.

Foley, R C and Singh, K B (1994). Isolation of a *Vicia faba* metallothionein like gene: Expression in foliar trichomes. *Plant Mol. Biol., 26*: 435-444. DOI: 410.1007/BF00039552.

Fowler, T J; Bernhardt, C and Tierney, M L (1999). Characterization and expression of four proline-rich cell wall protein genes in *Arabidopsis* encoding two distinct subsets of multiple domain proteins. *Plant Physiol., 121*: 1081-1091.

Franssen, H J; Nap, J P; Gloudemans, T; Stiekema, W; Van Dam, H; Govers, F; Louwerse, J; Van Kammen, A and Bisseling, T (1987). Characterization of cDNA for nodulin-75 of soybean: A gene product involved in early stages of root nodule development. *Proc. Natl. Acad. Sci. USA, 84(13)*: 4495-4499. DOI: 10.1073/pnas.84.13.4495.

Fujino, K; Obara, M and Sato, K (2014). Diversification of the plant-specific hybrid glycine-rich protein (*HyGRP*) genes in cereals. *Front. Plant Sci., 5*: 489-489. DOI: 10.3389/fpls.2014.00489.

García-Hernández, M; Murphy A and Taiz, L (1998). Metallothioneins 1 and 2 have distinct but overlapping expression patterns in *Arabidopsis*. *Plant Physiol., 118(2)*: 387-397. DOI: 10.1104/pp.118.2.387.

Guo, W J; Bundithya, W and Goldsbrough, P B (2003). Characterization of the *Arabidopsis* metallothionein gene family: Tissue-specific expression and induction during senescence and in response to copper. *New Phytol., 159*: 369-281.

Hernandez-Garcia, C M and Finer, J J (2014). Identification and validation of promoters and *cis*-acting regulatory elements. *Plant Sci., 217-218*: 109-119.

Ho, C-L; Tan, Y-C; Yeoh, K-A; Ghazali, A-K; Yee, W-Y and Hoh, C-C (2016). De novo transcriptome analyses of host fungal interactions in oil palm (*Elaeis guineensis* Jacq.). *BMC Genomics*, 17: 66.

Hsieh, P-H; Oyang, Y-J and Chen, C-Y (2019). Effect of *de novo* transcriptome assembly on transcript quantification. *Sci. Rep., 9(1)*: 8304. DOI: 10.1038/s41598-019-44499-3.

Jeong, J S; Kim, Y S; Baek, K H; Jung, H; Ha, S H; Choi, Y D; Kim, M; Reuzeau, C and Kim, J K (2010). Root-specific expression of *OsNAC10* improves drought tolerance and grain yield in rice under field drought conditions. *Plant Physiol. Biochem., 153*: 185-197.

Josè-Estanyol, M; Ruiz-Avila, L and Puigdomenech, P (1992). A maize embryo-specific gene encodes a proline-rich and hydrophobic protein. *Plant Cell, 4*: 413-423.

Joshi, R; Pareek, A and Singla-Pareek, S L (2016). Chapter 9 - Plant Metallothioneins: Classification, distribution, function, and regulation. *Plant Metal Interaction* (Ahmad, P ed.). Elsevier. p. 239-261.

Keller, B and Lamb, C J (1989). Specific expression of a novel cell wall hydroxyproline-rich glycoprotein gene in lateral root initiation. *Genes Dev., 3:* 1639-1646.

Kushairi, A; Ong-Abdullah, M; Nambiappan, B; Hishamuddin, E; Izzudin, Z B; Razmah, G; Subramaniam, V; Sundram, S and Parveez, G K A (2018). Oil palm economic performance in Malaysia and R&D progress in 2018. *J. Oil Palm Res., 31(2)*: 165-194.

Kushairi, A; Singh, R and Ong-Abdullah, M (2017). The oil palm industry in Malaysia: Thriving with transformative technologies. *J. Oil Palm Res., 29(4)*: 431-439.

Lalonde, E; Ha, K C H; Wang, Z; Bemmo, A; Kleinman, C L; Kwan, T; Pastinen, T and Majewski, J (2011). RNA sequencing reveals the role of splicing polymorphisms in regulating human gene expression. *Genome Res., 21(4)*: 545-554.

Li, Y; Li, C; Cheng, L; Yu, S; Shen, C and Pan, Y (2019). Over-expression of *OsPT2* under a rice root specific promoter *Os03g01700. Plant Physiol. Biochem., 136*: 52-57.

Lister, R; O'Malley, R C; Tonti-Filippini, J; Gregory, B D; Berry, C C; Millar, A H and Ecker, J R (2008). Highly integrated single-base resolution maps of the epigenome in *Arabidopsis*. *Cell, 133*: 523-536.

Liu, J J and Ekramoddoullah, A K (2003). Root-specific expression of a western white pine *PR10* gene is mediated by different promoter regions in transgenic tobacco. *Plant Mol. Biol., 52:* 103-120. DOI: 10.1023/A:1023930326839.

Liu, Q; Ding, C; Lang, X; Guo, G; Chen, J and Su, X (2019). Small non-coding RNA discovery and profiling with sRNA tools based on high-throughput sequencing. *Brief. Bioinform., 22(1)*: 463-473. DOI: 10.1093/bib/bbz151.

Livak, K J and Schmittgen, T D (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) method. *Methods, 25(4)*: 402-408. DOI: 10.1006/meth.2001.1262.

Low, E T L; Jayanthi, N; Chan, K-L; Sanusi, N S N M; Halim, M A A; Rosli, R; Azizi, N; Amiruddin, N; Angel, L P L; Ong-Abdullah, M; Singh, R; Manaf, M A A; Sambanthamurthi, R; Parveez, G K A and Kushairi, A (2017). The oil palm genome revolution. *J. Oil Palm Res., 29(4)*: 456-468.

Lu, G and Moriyama, E N (2004). Vector NTI, a balanced all-in-one sequence analysis suite. *Brief. Bioinform., 5(4):* 378-388. DOI: 10.1093/bib/5.4.378.

Masani, M Y A; Izawati, A M D; Omar, O A and Parveez, G K A (2018). Biotechnology of oil palm: Current status of oil palm genetic transformation. *Biocatal. Agric. Biotechnol., 15*: 335-347.

Masura, S S; Tahir, N I; Rasid, O A; Ramli, U S; Othman, A; Masani, M Y A; Parveez, G K A and Kushairi, A (2017). Post-genomic technologies for the advancement of oil palm research. *J. Oil Palm Res., 29(4)*: 469-486.

Meister, R; Rajani, M S; Ruzicka, D and Schachtman, D P (2014). Challenges of modifying root traits in crops for agriculture. *Trends Plant Sci., 19*: 779-788.

Morris, E P; Chan, P K; Ooi, C L L; Ong, P W; Kamaruddin, K; Marjuni, M; Amiruddin, M A; Chan, K L; Rosli, R; Low, E T L; Singh, R; Shaharuddin, N A; Murphy, D J; Manaf M A A and Siti Nor Akmar, A (2020). Transcriptome analysis across various mesocarp developmental stages of MPOB-Angola *Dura*. *J. Oil Palm Res., 32(3)*: 406-419.

Naher, L; Yusuf, U K; Ismail, A; Tan, S G and Mondal, M M A (2013). Ecological status of *Ganoderma* and basal stem rot disease of oil palms (*Elaeis guineensis* Jacq.). *Aust. J. Crop Sci., 7(11)*: 1723-1727.

Nitz, I; Berkefeld, H; Puzio, P S and Grundler, F M V (2001). *Pyk10*, a seedling and root specific gene and promoter from *Arabidopsis thaliana. Plant Sci., 161*: 337-346.

Oikonomopoulos, S; Bayega, A; Fahiminiya, S; Djambazian, H; Berube, P and Ragoussis, J (2020). Methodologies for transcript profiling using long-read technologies. *Front. Genet., 11*: 606. DOI: 10.3389/fgene.2020.00606.

O'Leary, N A; Wright, M W; Brister, J R; Ciufo, S; Haddad, D; McVeigh, R; Rajput, B; Robbertse, B; Smith-White, B; Ako-Adjei, D; Astashyn, A; Badretdin, A; Bao, Y; Blinkova, O; Brover, V; Chetvernin, V; Choi, J; Cox, E; Ermolaeva, O; Farrell, C M; Goldfarb, T; Gupta, T; Haft, D; Hatcher, E; Hlavina, W; Joardar, V S; Kodali, V K; Li, W; Maglott, D; Masterson, P; McGarvey, K M; Murphy, M R; O'Neill, K; Pujar, S; Rangwala, S H; Rausch, D; Riddick, L D; Schoch, C; Shkeda, A; Storz, S S; Sun, H; Thibaud-Nissen, F; Tolstoy, I; Tully, R E; Vatsan, A R; Wallin, C; Webb, D; Wu, W; Landrum, M J; Kimchi, A; Tatusova, T; DiCuccio, M; Kitts, P; Murphy, T D and Pruitt, K D (2016). Reference sequence (RefSeq) database at NCBI: Current status, taxonomic expansion, and functional annotation. *Nucleic Acids Res., 44(D1)*: D733-745. DOI: 10.1093/nar/gkv1189.

Parveez, G K A; Rasid, O A; Masani, M Y A and Sambanthamurhi, R (2015). Biotechnology of oil palm: Strategies towards manipulation of lipid content and composition. *Plant Cell Rep., 34*: 533-543.

Potenza, C; Aleman, L and Sengupta-Gopalan, C (2004). Targeting transgene expression in research, agricultural and environmental applications: Promoters used in plant transformation. *In Vitro Cell. Dev. Biol. Plant, 40*: 1-22.

Pruitt, K D; Tatusova, T and Maglott, D R (2005). NCBI Reference Sequence (RefSeq): A curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res., 33 (Database issue)*: D501-D504. DOI: 10.1093/nar/gki025.

Rasid, O A; Masura, S S; Hanin, A N; Izawati, A M D; Bohari, B; Masani, M Y A and Parveez, G K A (2020). Oil palm transgenic research: Challenges, update and future outlook. *The oil palm genome. Compendium of Plant Genome* (Ithnin, M and Kushairi, A eds.). Springer, Cham. p. 69-81. DOI: 10.1007/978-3-030-22549-0_6.

Reid, S J and Ross, G S (1997). Upregulation of two cDNA clones encoding metallothionein-like proteins in apple fruit during cool storage. *Physiol. Plant., 100*: 183-189. DOI: 110.1111/j.1399-3054.1997.tb03471.x.

Rival, A (2017). Breeding the oil palm *(Elaeis guineensis* Jacq.) for climate change. *OCL-Oilseeds Fats Crops Lipids, 24*: D107.

Rivas, M A; Beaudoin, M; Gardet, A; Stevens, C; Sharma, Y; Zhang, C K; Boucher, G; Ripke, S; Ellinghaus, D; Burtt, N; Fennell, T; Kirby, A; Latiano, A; Goyette, P; Green T; Halfvarson, J; Haritunians, T; Korn, J M; Kuruvilla, F; Lagacé, C; Neale, B; Lo, K S; Schumm, P and Törkvist, L (2011). Deep resequencing of GWAS loci identifies independent rare variants associated with inflammatory bowel disease. *Nat. Genet., 43*: 1066-1073.

Sanders, R; Mason, D J; Foy, C A and Huggett, J F (2014). Considerations for accurate gene expression measurement by reverse transcription quantitative PCR when analysing clinical samples. *Anal. Bioanal. Chem., 406(26)*: 6471-6483. DOI: 10.1007/s00216-014-7857-x.

Shabb, J B; Muhonen, W W and Mehus, A A (2017). Quantitation of human metallothionein isoforms in cells, tissues and cerebrospinal fluid by mass spectrometry. *Methods Enzymol., 586*: 413-431.

Showalter, A M and Rumeau, D (1990). Molecular biology of the plant cell wall hydroxyproline-rich glycoproteins. *Organization and Assembly of Plant and Animal Extracellular Matrix* (Adair, W S and Mecham, R P eds.). Academic Press, New York. p. 247-281.

Singh, R; Meilina, O A; Low, E T L; Arif, M A A; Rozana, R; Rajanaidu, N; Ooi, C L L; Ooi, S E; Chan, K L; Mohd Amin, H; Norazah, A; Jayanthi, N; Bacher, B; Lakey, N; Smith, S W; He, D; Hogan, M; Budiman, M A; Lee, E K; Desalle, R; Kudrna, D; Goicoechea ,J L; Wing, R A; Wilson, R K; Fulton, R S; Ordway, J M; Martienssen, R A and Sambanthamurthi, R (2013). Oil palm genome sequence reveals divergence of interfertile species in old and new worlds. *Nature, 500(7462)*: 335-339.

Srinath, T; Reddy, V D and Rao, K V (2017). Isolation and functional characterization of a novel stress inducible promoter from pigeon pea (*Cajanus cajan* L). *Plant Cell Tiss. Organ Cult., 128*: 457-468.

Suzuki, H; Wagner, T and Tierney, M L (1993). Differential expression of two soybean *(Glycine max* L.) proline-rich protein genes after wounding. *Plant Physiol., 101*: 1283-1287.

Tikhonovich, I A and Provorov, N A (2007). Chapter 14 - Beneficial plant-microbe interactions. *Comprehensive and Molecular Phytopathology* (Dyakov, Y T; Dzhavakhiya, V G and Korpela, T eds.). Elsevier, Amsterdam. p. 365-420.

Trapnell, C; Pachter, L and Salzberg, S L (2009). TopHat: Discovering splice junctions with RNA-Seq. *Bioinformatics, 25:* 1105-1111.

Trapnell, C; Williams, B A; Pertea, G; Mortazavi, A; Kwan, G; van Baren, M J; Salzberg, S T; Wold, B J and Pachter, L (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol., 28(5)*: 511-515. DOI: 10.1038/nbt.1621.

Wasson, A P; Richards, R A; Chatrath, R; Misra, S C; Prasad, S V and Rebetzke, G J (2012). Traits and selection strategies to improve root systems and water uptake in water-limited wheat crops. *J. Exp. Bot., 63*: 3485-3498. DOI: 3410.1093/jxb/ers3111.

Wilson, R C; Long, F; Maruoka, E M and Cooper, J B (1994). A new proline-rich early nodulin from *Medicago truncatula* is highly expressed in nodule meristematic cells. *Plant Cell, 6*: 1265-1275.

Winicov, I; Valliyodan, B; Xue, L and Hoober, J K (2004). The *MsPRP2* promoter enables strong heterologous gene expression in a root-specific manner and is enhanced by overexpression of Alfin 1. *Planta, 219*: 925-935.

Xu, Y; Buchholz, W G; DeRose, R T and Hall, T C (1995). Characterization of a rice gene family encoding root-specific proteins. *Plant Mol. Biol., 27*: 237-248.

Xue, G-P; Rae, A L; White, R G; Drenth, J; Richardson, T and McIntyre, C L (2016). A strong root-specific expression system for stable transgene expression in bread wheat. *Plant Cell Rep., 35(2)*: 469-481.

Yamamoto, Y T; Taylor, C G; Acedo, G N; Cheng, C L and Conkling, M A (1991). Characterization of *cis*-acting sequences regulating root-specific gene expression in tobacco. *Plant Cell, 3*: 371-382.

Yeap, W C; Loo, J M; Wong, Y C and Kulaveerasingam, H (2014). Evaluation of suitable reference genes for qRT-PCR gene expression normalization in reproductive, vegetative tissues and during fruit development in oil palm. *Plant Cell Tissue Organ Cult., 116(1)*: 55-66. DOI: 10.1007/s11240-013-0382-3.

Zeng, Y and Yang, T (2002). RNA isolation from highly viscous samples rich in polyphenols and polysaccharides. *Plant Mol. Biol. Rep., 20*: 417a-417e.

Zubaidah, R and Siti Nor Akmar, A (2005). The effects of metal ions on root-specific expression of the oil palm *MT3-B* gene promoter. *Proc. of the PIPOC 2005 International Palm Oil Congress-Agriculture Conference.* MPOB, Bangi. p. 1104-1110.

Zulkifli, Y; Norziha, A; Naqiuddin, M H; Fadila, A M; Nor Azwani, A B; Suzana, M; Samsul, K R; Ong-Abdullah, M; Singh, R; Parveez, G K A and Kushairi, A (2017). Designing the oil palm of the future. *J. Oil Palm Res., 29*: 440-455.

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EFFECTIVENESS OF *Bacillus thuringiensis* AERIAL SPRAYING AGAINST THE BAGWORM, *Metisa plana* WALKER (Lepidoptera: Psychidae) OUTBREAK IN OIL PALM USING DRONE

MOHAMED MAZMIRA MOHD MASRI[1]*; NOORHAZWANI KAMARUDIN[1]; NUR ROBAATUL MOHD ALI NAPIAH[1] and MOHD FAHMI KENI[1]

ABSTRACT

Bagworms have been a severe threat to the oil palm industry and have endangered mostly smallholdings. Thus, aerial spraying of Bacillus thuringiensis *(Bt) based bioinsecticides using an agricultural drone was carried out to control the bagworm infestation at a severely infested oil palm smallholding located in Sepang, Selangor, Malaysia. The drone was equipped with VP110 nozzle at the pressure of 0.2 MPa with a permanent speed of 2.8 m s^{-1}. The flying height was set 2 m above the canopy. The first round of drone spraying conducted on 4 July 2017 has successfully reduced the larval population of the bagworm,* Metisa plana *from 304.5 larvae per frond (LPF) at 0 days after treatment (DAT) to 12.1 LPF at 28 DAT, resulting to 96% reduction in the larval numbers. However, some of the larvae survived to the pupal stage, with approximately 64.52 pupae per frond (PPF) recorded at 28 DAT. The second drone spraying conducted on 27 August 2017 has effectively decreased the larval number from 109.25 LPF at 0 DAT to 9.3 LPF at 28 DAT, resulting in 91.5% of larvae reduction with zero pupae recorded. The results showed a great potential of drone aerial spraying in controlling bagworms at oil palm planting areas.*

Keywords: bagworm infestation, biopesticides, drones, unmanned aerial vehicles.

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INTRODUCTION

In Malaysia, two bagworm species, namely *Metisa plana* and *Pteroma pendula* have caused severe yield losses up to 33%-47% in oil palm with *M. plana* being the most economically significant and important defoliator (Basri, 1993; Ramlah *et al*., 2007a). Bagworm outbreaks have been reported to be more severe in the 1990s until present (Wood and Kamarudin, 2019) in which the infestations had seriously affected the yield of oil palm due to procrastinated and incorrect control strategies (Tey and Cheong, 2013). The outbreaks of these leaf-eating caterpillars which brings about severe losses to the oil palm industry therefore need to be prevented (Norman and Mazmira, 2019). The invasion of these leaf eating pests has affected the majority of oil palm smallholders, making it a national concern (Thomas, 2017). In 2017, more than 30 000 ha of oil palm smallholdings was infested by bagworm (Kamarulbaid, 2018). The recurrence of the bagworm outbreak in the oil palm industry has therefore contributed to severe economic losses of more than USD25 million annually, and urgent control measures should be undertaken. Without proper control, severely attacked palms can suffer high crop loss, which futher affect the livelihoods of oil palm planters, especially the smallholders. On 15 November 2013, under the Malaysia Act 167 (Plant

[1] Malaysian Palm Oil Board,
6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.

* *Corresponding author e-mail: mazmira@mpob.gov.my*

Quarantine Act 1967), the Malaysian government declared bagworm as a dangerous pest (Kamarudin *et al*., 2017). Under the Plant Quarantine Act 1967, any plantation owner or smallholder who fails to take action in controlling the bagworm infestation will be penalised not more than RM10 000 or jail for two years (Attorney General's Chambers, 2013).

Control measures using chemical insecticides application have been widely conducted in plantations compared to smallholdings (Hasber, 2010). The application of broad-spectrum chemical insecticides such as cypermethrin will cause a direct effect in reducing the population of natural enemies (parasitoid and predators) of the bagworms, hence, increasing the risk of an outbreak (Norman and Mazmira, 2019). Biological control developed from natural agents that provides long term control of the bagworm outbreak. Aerial spraying using a *Bacillus thuringiensis* (Bt) is one of the viable approaches for controlling bagworms in oil palm. Bt is an environmentally friendly microbial insecticide and does not cause harm to freshwater fish and other living organisms (Ahmad *et al*., 2014; 2015).

Usage of aircraft for aerial spraying is one of the best strategies to control a large outbreak area. In China, agricultural aerial spraying effectively ensures that food and ecological security is achieved (Zhang *et al*., 2018). In Malaysia, an aerial spraying operation carried out in Slim River, Perak for controlling *M. plana* has successfully reduced the overall population of bagworms to below the threshold level (Kamarudin *et al*., 2017). However, usage of agricultural aircraft has many challenges, including the aircraft's technical issues, availability of airstrips and permit approvals from the Civil Aviation Authority of Malaysia (CAAM) and Department of Agriculture (DOA) which may cause delays in controlling the bagworm outbreaks. An agricultural aircraft had crashed during aerial spraying of Bt for bagworm control in Pahang on 25 February 2018 due to technical issues (Amin, 2018).

In order to reduce the dependence on agricultural aircraft, the focus has now been shifted to the usage of lightweight, unmanned aerial vehicles (UAVs), commonly known as drones. Drones are remote-controlled aircrafts with no human pilots on board. Drones have a huge potential in supporting evidence-based planning and in spatial data collection. Most applications of drone technology depend on their ability to generate and deliver accurate information (European Commission, 2018). Despite some intrinsic restrictions, these tools and technologies can deliver valuable data that can then be used for control strategies and making vital decisions. Drones are usually operated remotely by telemetry through autonomous flight along pre-programmed paths using global positioning system (GPS) or visual contact by the operator (Giles and Billing, 2015). The first uses of drones in agriculture have mostly focussed on remote sensing and visual inspection of crop or field surroundings. In the present era, drone application has been widely used in agriculture and serves as an alternative to manned aircraft. Drones have also assisted in the technical analysis for precision agriculture, such as crop monitoring and crop height where it was found that UAV increased the productivity of spraying activity and optimised the usage of water and chemicals (Rao Mogili and Deepak, 2018). A study by Giles and Billing (2015) shows that UAV was successfully utilised in a vineyard spraying operation where the workload reached up to 50 L ha^{-1} with a coverage of between 2-5 ha hr^{-1}. UAV technology has also shown substantial improvements in crop scouting, yield and field boundary mapping, pest control and spraying. UAVs which can also be deployed promptly and repeatedly, are safer than aircraft, and have flexible timing of missions.

Paddy monitoring using a multirotor UAV and RGB digital camera in Kelantan was reported by Norasma (2018). Mat Su *et al*. (2018) reported that aerial spraying using a drone is comparable to knapsack spraying with coefficient values (CVs) of 0.46 and 0.43, respectively, with uniformity of spraying at 2 m height. The Muda Agricultural Development Authority (MADA) has also carried out commercial chemical pesticide spraying using drones on 2000 ha of paddy (Bernama, 2017). Nevertheless, no technical paper has been published on UAV operation and efficacy. Besides paddy, no other crops have been reported to utilise drone technology for aerial spraying.

The potential of aerial spraying using drones in the oil palm industry is enormous, especially for controlling oil palm pests such as bagworms. The objective of this research was to determine the effectiveness of aerial spraying of Bt using drones against the bagworm, *M. plana* in oil palm plantation.

MATERIALS AND METHODS

Bacillus thuringiensis (Bt) Based Biopesticides for the Aerial Spray Activity

Bt was mass-produced using liquid-state fermentation and laboratory prepared medium at Malaysian Palm Oil Board (MPOB) Microbial Technology and Engineering Centre (MICROTEC) in 5-500 L bioreactors (Sartorius Stedim, Germany) for 48 hr, at 30°C (Ahmad *et al*., 2012). The mass production of microbial insecticide based on local isolates known as MPOB Bt1 for controlling bagworm has been patented (Patent No. PI2011000307) (Ramlah *et al*., 2011). Ecobac-1 (EC) is an emulsified product derived from an indigenous

strain of Bt (MPOB Bt1) (Ramlah and Basri, 1997). The active ingredients of EC are spores and δ-endotoxins, standardised to 1600 IU mg^{-1}.

The UAV (Drone) System and Specifications

The drone used for the spraying operation was JMR V1650 measuring 1650 mm in diagonal length (*Figure 1*). The drone was powered by a twin pack of 22 000 mAh 22.2 V lithium polymer battery connected in series and can carry a payload of up to 20 L of pesticides. The maximum take-off weight is 38 kg and it can fly with a full payload for around 10-13 min. With the arms fully extended, the spraying width can go up to 8 m. With a total of four nozzles, the biopesticide can be sprayed at 1.8 L min^{-1}. The flying height of the drone during spraying, was at 2-3 m from the palm canopy for better droplet distribution on the palm leaves. Other specifications of the drone as listed in *Table 1.*

Figure 1. The drone, JMR V1650 used for the aerial spraying operation.

TABLE 1. SPECIFICATIONS OF THE DRONE, JMR-V1650 FOR THE SPRAYING OPERATION OF BAGWORM

Parameters	Value/ information
Solution volume	15 L
Nozzle model	VP 110 015
Pump pressure	0.2 MPa
Flow rate	1.8 L min^{-1} (0.45 L min^{-1} x 4 nozzles)
Working flight speed	1.9-2.2 m s^{-1}
Spray operation time	8-10 min
Flying height	2-3 m above canopy
Area covered	0.5 ha
Bt application rate	30 L ha^{-1}
Bt rate	1.5 L ha^{-1}
Flight mode	ATT/Spray work/GPS
Maximum flight height	30 m (for automated operation)
Flight operation time	20 min maximum (take-off to landing)
Control distance	1 000 m maximum

Sprayer Module

The 110° fan nozzle nozzle size was 0.4 mm (VP 110015). The distance between the two nozzles was 50 cm. The variable pressure nozzle maintains a consistent spray angle over a wide pressure range, up to 20 psi (1 bar) and is available in 80° and 110° versions to work with different boom heights. At 0.2 MPa, the flow rate is at 0.45 L min^{-1}. A total of 0.5 ha area was covered for each flight with working flight speed ranging from 1.9-2.2 m s^{-1} and solution volume of 15 L. The volume application rate was 30 L ha^{-1} at a biopesticide rate of 1.5 L ha^{-1}. The detailed specification of the spraying is as shown in *Table 1*.

Drone Spraying Field Operation

The first drone spraying was conducted against the susceptible first to fourth larval instars of *M. plana* at Kg. Simpang Morib, Selangor, Malaysia smallholdings on 4 July 2017. The drone spraying was scheduled based on the life cycle of the bagworm. The average life cycle for *M. plana* from eggs to adults takes approximately 103.5 days (Kok *et al.*, 2011). Due to the multi-stage infestation of bagworms in the area, some larvae had survived to the pupal stage at 28 days after treatment (DAT). Hence, the second round of drone spraying was carried out on 27 August 2017. The infested area was GPS-mapped (*Figure 2*) and sprayed with 0.75 L EC diluted in the drone tank using 15 L of water. The total area sprayed was 5.56 ha. In both aerial spraying operations, an adjacent area without any treatment was set up as a control plot.

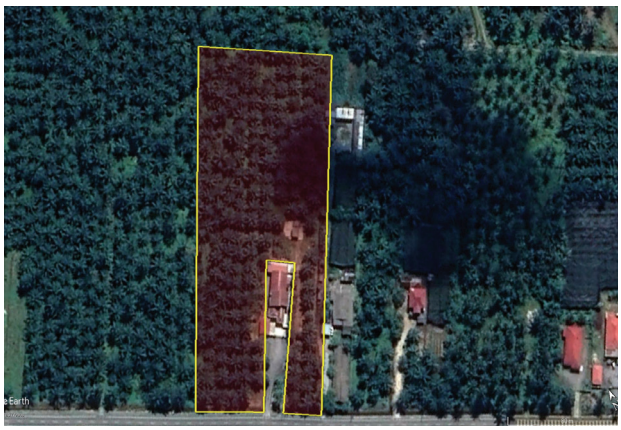


Figure 2. Aerial map of the oil palm smallholdings in Sepang affected by bagworm.

Bagworms Census

A pre-census of the bagworm population was conducted before aerial spraying operation to count and record the initial bagworm population.

Post-census counts of the bagworm populations were conducted at 7, 14 and 28 DAT. One percent of the infested area was censused by taking one palm at every 10th palm at every 10th row. Frond number 17 from the middle of the canopy showing fresh damage symptoms was cut down for counting, and the number of larvae and pupae on both sides of the frond was recorded (MPOB, 2016). The control procedures must be conducted once the eggs within the female pupal bags hatch into early larval stages (Norman *et al*., 2004; Ramlah *et al*., 2007a; 2007b). Control measures should start immediately when the larval population is at the early instar stages (first to fourth larval instars), and the number is above the threshold level [5-10 larvae per frond (LPF)] (Wood, 2002). If more than 70% of the population are at the late instars (fifth to seventh larval instars) or pupal stages, the aerial spray using Bt must be postponed (Basri, 1993; Ramlah *et al*., 2007a; 2007b). The aerial spray would then be carried out on the next generation of *M. plana* once the early larval stages emerge.

Water Sensitive Paper (WSP) Location and Droplet Distribution

Spray droplets were determined using WSP during each operation. WSPs were placed on rectangular wooden boards attached to a pole in the middle of the harvesting path. The heights of the poles were adjusted to match the heights of the oil palm trees (MPOB, 2016). One pole was divided into three levels with 1.0 m space for each level beginning from the same level with oil palm height, and each level was placed with four WSP for a total of 12 WSPs per pole (*Figure 3*). One pole was positioned at every 30 ha of the target area. These WSPs would indicate and ensure that the biopesticides mist is well distributed during the spraying operation. After every spraying operation, the WSPs will be collected immediately. After the droplets dried, the WSP was placed in marked envelopes, stored in sealed dry plastic bags and transferred to the laboratory for further analysis. The DropLeaf app was used to analyse and calculate the droplet distribution on WSPs. The volume median diameter (VMD) was also analysed in the first and second round of the aerial sprayings. VMD stands for midpoint droplet size (median), where half of the spray volume is in smaller droplets and half of the volume is in bigger droplets.

Data Analysis

Data on the field survival of bagworms treated with the Bt1 product was analysed using two-way analysis of variance (ANOVA) (Sigma-plot version 12.5) with the main factor was treatment plot (Bt treatment plot and control plot) and subfactor was the DAT interval (0, 7, 14, 21 and 28 DAT). The means were separated using the Tukey comparison test. The time course population dynamics of the different stages of bagworms was closely monitored for precision in follow-up sprays.

RESULTS

Bagworm Mortality During the First Round of Drone Spraying

The application of drone aerial spraying for controlling multi-stage bagworm outbreaks was carried out in the hotspot area at an oil palm smallholding in Sepang, Selangor, Malaysia. The first round of drone spraying had commenced on 4 July 2017. At 0 DAT, the data showed that the

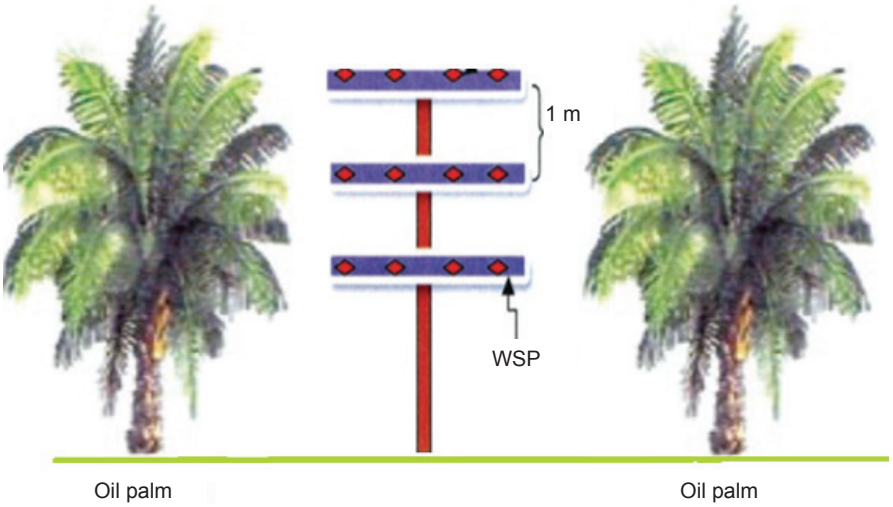


Figure 3. The position of the pole with WSP during the aerial spraying.

overall mean LPF was 304.5, with the majority of the larvae at larval instars 3 (L3) (*Table 2*). The mean population of mature larvae (L4 and L5) was approximately 117.7 and 25.5 LPF, respectively. The high number of mature larvae indicated that there was a high probability of the population surviving the treatment once it entered the pupal stage. Seven days after the treatment, the percentage of larval reduction recorded was moderate (30.6%) with the overall average of 211.2 LPF. Significant reduction in the mean larval population was recorded starting at 14 DAT, where the pooled average larval population decreased from 304.5-149.9 LPF (*Table 2*) which was 50.7% reduction in larval population.

Larval reduction was further recorded at 21 DAT with an overall mean of 74.7 LPF or 75.5% in larval reduction. At 28 DAT, the overall mean of larval population had decreased substantially to 12.1 LPF with 96% reduction in larval population. Although the overall mean of LPF recorded at 28 DAT was small, the overall mean pupae per frond (PPF) was significantly high at 64.5 PPF. The data showed that drone spraying using Bt has effectively reduced the number of larvae; however, due to the severe and multi-stage infestation in the area, some of the mature larvae had managed to enter the pupa stage. Thus, repetition of treatment was required to control the second generation of bagworms from the survived pupae.

In contrast, the bagworm population in the untreated control plot recorded a high population of the total mean number of bagworms ranging from 131 bagworms per frond (BPF) at 0 DAT to 137 BPF at 28 DAT (*Table 3*). There was no reduction of the total bagworm number in the control plot due to natural factors because the area was concomitantly being attacked by multi-stage bagworm infestation. The total mean number of bagworms recorded in the control plot maintained above the economic injury threshold level until 28 DAT.

TABLE 2. EFFECT OF ECOBAC-1 (EC) AGAINST BAGWORM SURVIVAL BEFORE AND AFTER THE FIRST ROUND OF DRONE SPRAY

Bagworm stage	0 DAT (4/7/17)	7 DAT (10/7/17)	14 DAT (17/7/17)	21 DAT (24/7/17)	28 DAT (31/7/17)
	Mean ± SE				
L1	0	0	0	0	0
L2	38.62 ± 2.9	0	0	0	0
L3	122.72 ± 10.0	37.30 ± 3.0	0	0	0
L4	117.70 ± 10.7	74.34 ± 6.8	19.08 ± 1.2	0	0
L5	25.46 ± 4.2	73.74 ± 6.7	60.14 ± 2.6	14.62 ± 1.6	0
L6	0	25.82 ± 2.9	54.66 ± 2.5	25.60 ± 2.0	1.80 ± 0.2
L7	0	0	16.10 ± 1.2	34.52 ± 2.0	10.30 ± 0.8
Overall mean pupae (per frond)	0	0	0	11.74 ± 1.2	64.52 ± 2.2
Overall mean larvae (per frond)	304.50 ± 21.4^{a}	211.20 ± 15.0^{b}	149.98 ± 6.0^{c}	74.74 ± 4.5^{d}	12.10 ± 0.8^{e}
Overall mean bagworm (per frond)	304.50 ± 21.4^{a}	211.20 ± 15.0^{b}	149.98 ± 6.0^{c}	86.48 ± 4.9^{d}	76.62 ± 2.6^{e}
% Larvae reduction	-	30.6	50.7	75.5	96.0
% Bagworm reduction	-	30.6	50.7	71.6	74.8

Note: L indicates bagworm larval instars. Mean with the same letters in the same row are not significantly different by Tukey comparison test ($p<0.001$).

TABLE 3. COMPARISON OF THE TOTAL MEAN NUMBER OF BAGWORM IN THE CONTROL AND Bt TREATMENT PLOT DURING THE FIRST ROUND OF DRONE SPRAY

	DAT	Total bagworm per frond (BPF)	
		Control plot	Bt treated plot
No. of bagworm (Mean ± SE)	0	131.06 ± 3.83 Ac	304.50 ± 21.4 Ba
	7	133.40 ± 2.62 Ac	211.20 ± 15.0 Bb
	14	137.16 ± 2.45 Ac	149.98 ± 6.0 Bc
	21	132.50 ± 2.45 Ac	86.48 ± 4.9 Bd
	28	136.98 ± 2.23 Ac	76.62 ± 2.6 Bd

Note: Means that do not share a capital/small letter in the table are significantly different. Tukey simultaneous test at 95% confidence levels.

Bagworm Mortality During the Second Round of Drone Spraying

The second round of drone spraying was carried out on 27 August 2017. At 0 DAT, overall mean recorded was 109.3 LPF. Majority of the larvae were still at L1 and L2, whilst none were recorded from L3 to L7. There were no pupae recorded at 0 DAT. Similar to the first drone spraying, the percentage of larval reduction at 7 DAT was recorded under 50.0%, which was only 27.5% reduction (*Table 4*). The overall mean recorded was 79.2 LPF. A significant reduction in the bagworm population was recorded at 14 DAT. The percentage of larval reduction recorded at 14 DAT was more than 50.0% with an overall mean of 52.6 LPF. The overall mean of LPF kept decreasing at 21 DAT with 25.3 LPF and 76.8% larval reduction. Eventually, at 28 DAT, the larval population was recorded under the threshold level with only 9.3 LPF. The final percentage of larvae reduction at 28 DAT was 91.5%. No pupae were recorded at 28 DAT. With the right strategy, the drone spraying had successfully controlled the bagworm outbreak in just two rounds of drone spraying application.

In the control plot, the total mean number of bagworms had increased tremendously after more than a month left untreated. At 0 DAT, the total mean was 472 BPF, almost three times higher than the number recorded at 28 DAT in the control plot during the first drone aerial spray (*Table 5*). The bagworm number increased to 501 BPF at 28 DAT, severely damaging the oil palms in the area. The data clearly showed that if the bagworm attack was left untreated as in the control plot, the bagworm population will keep increasing and will severely affect the oil palm trees.

The unequal number of bagworm populations in the control and treated plots in the zero-day for the first spray was due to the selection of control plot that had low bagworm population to avoid severe effects at that area. Due to no treatment at the control plot, the bagworm population was different compared to the treated area in the zero-days for the second spray.

TABLE 4. EFFECT OF ECOBAC-1 (EC) AGAINST BAGWORM SURVIVAL BEFORE AND AFTER THE SECOND ROUND OF DRONE SPRAY

Bagworm stage	0 DAT (27/8/17)	7 DAT (5/9/17)	14 DAT (11/9/17)	21 DAT (18/9/17)	28 DAT (25/9/17)
	Mean ± SE				
L1	35.25 ± 0.91	0	0	0	0
L2	74.00 ± 2.27	23.35 ± 2.32	0	0	0
L3	0	52.80 ± 2.62	12.65 ± 0.91	0	0
L4	0	3.05 ± 0.74	32.50 ± 1.35	5.75 ± 0.53	0
L5	0	0	7.45 ± 0.82	17.20 ± 0.73	1.40 ± 0.18
L6	0	0	0	2.35 ± 0.40	6.65 ± 0.18
L7	0	0	0	0	1.25 ± 0.19
Overall mean pupae (per frond)	0	0	0	0	0
Overall mean larvae (per frond)	109.25 ± 2.87^{a}	79.20 ± 4.41^{a}	52.60 ± 2.56^{b}	25.30 ± 0.71^{c}	9.30 ± 0.28^{d}
Overall mean bagworm (per frond)	109.25 ± 2.87^{a}	79.20 ± 4.41^{a}	52.60 ± 2.56^{b}	25.30 ± 0.71^{c}	9.30 ± 0.28^{d}
% Larvae reduction	-	27.5	51.9	76.8	91.5
% Bagworm reduction	-	27.5	51.9	76.8	91.5

Note: L indicates bagworm larval instars. Mean with the same letters in the same row are not significantly different based on Tukey comparison test (p=<0.001).

TABLE 5. COMPARISON OF THE TOTAL MEAN NUMBER OF BAGWORM IN THE CONTROL AND Bt TREATMENT PLOT DURING THE SECOND ROUND OF DRONE SPRAY

	Time (DAT)	Total bagworm per frond (BPF)	
		Control plot	Bt treated plot
No. of bagworm (Mean ± SE)	0	472.30 ± 5.60 Ab	109.25 ± 2.87 Bc
	7	468.40 ± 2.10 Ab	79.20 ± 4.41 Bd
	14	469.55 ± 1.10 Ab	52.60 ± 2.56 Be
	21	474.75 ± 1.93 Ab	25.30 ± 0.71 Bf
	28	501.05 ± 2.93 Aa	9.30 ± 0.28 Bg

Note: Means that do not share a capital/small letter in the table are significantly different. Tukey simultaneous test at 95% confidence levels.

Droplets Distribution

The quality of spray application in the field is usually measured by WSPs or Kromekote® card attached to selected target areas or leaves and inspected after the spraying operation (Sundaram *et al*., 1987; Theriault *et al*., 2001). The results will indicate the average amount of droplets deposited according to the flight height and velocity contributed during the drone spraying operation. In the current study, the droplets had distributed uniformly on the leaves. The average droplet deposition and uniformity of each spraying operation were as shown in *Figure 4*. During the first round of spraying, the droplets characteristics were sparse while in the second round of spraying the droplets were slightly denser (*Figure 4*). Each pole consists of six WSPs. The number of droplets on each WSP card was analysed using DropLeaf apps. The average number of spray droplets deposited on the WSPs for the first-round sprays were 10.00-20.00 droplets cm^{-2} with VMD range of 710-774 μm while the second rounds sprays were 27.43-41.29 droplets cm^{-2} with VMD range of 732-790 μm (*Table 6*). Syngenta Crop Protection AG (Basel, Switzerland) recommends at least 20.00-30.00 droplets cm^{-2} for insecticide or pre-emergence herbicide applications, 30.00-40.00 droplets cm^{-2} for contact post-emergence herbicide applications, and 50.00-70.00 droplets cm^{-2} for fungicide applications to provide satisfactory results.

The droplets deposition during both operations showed that medium layer (ML) and bottom layer (BL) of WSP for both rounds captured less droplets compared to upper layer (UL) WSPs. Based on the bagworm census, it was found that the droplets uniformity is a good indicator or guide to evaluate the efficacy of the aerial spraying (Guo *et al*., 2019). The results also indicated that the flight height and velocity established in this operation was apposite for the uniform deposits of the droplets observed on the oil palm leaves.

Effect of Rainfall on Drone Spraying Efficacy and Bagworm Mortality

During the first round of drone spraying, rainfall was recorded at 3 DAT (6.6 mm), and the situation continued for three more days, with an average rainfall of 23.2 mm, 1.9 mm and 24.5 mm, respectively (*Figure 5*). The highest rainfall (35.1 mm) was recorded at 10 DAT. Beginning 12 DAT,

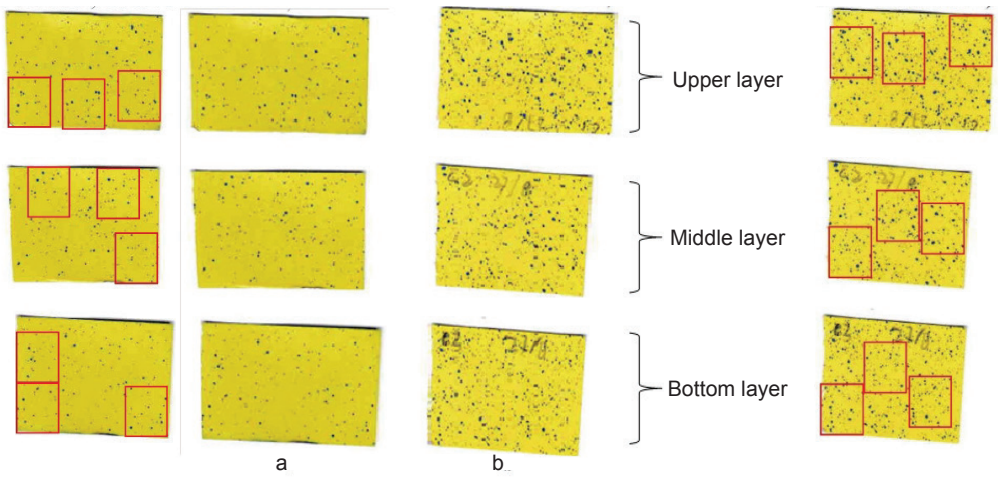


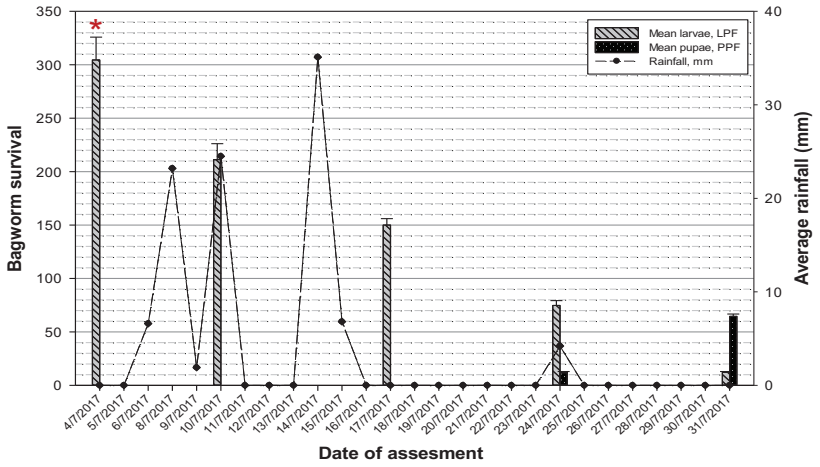
Figure 4. Droplet depositions on WSP placed on a pole at oil palm plantation during the drone spraying. (a) first round of drone spraying, and (b) second round of drone spraying.

TABLE 6. SUMMARY OF WSP SPRAY COVERAGE AT THREE POSITIONS IN THE CANOPY (Upper, Middle and Bottom) AT TWO ROUNDS OF SPRAYS

		Upper	Middle	Bottom
First round spray	WSP coverage area (%)	98.61	98.09	98.98
	Droplets cm^{-2}	20.00	15.86	10.00
	VMD (Dv 0.5 μm)	714.52	774.15	710.84
Second round spray	WSP coverage (%)	97.49	97.47	97.04
	Droplets cm^{-2}	41.29	32.14	27.43
	VMD (Dv 0.5 μm)	732.46	764.56	790.79

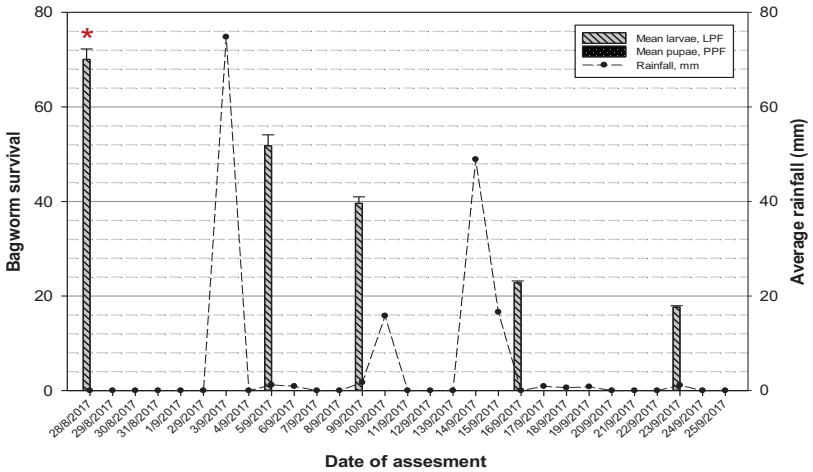
no rainfall was recorded until 28 DAT. Based on the results, it appears that rainfall did not significantly affect the drone spraying efficacy in controlling the bagworm population. The untimely rainfall only after 3 DAT and high rainfall at 10 DAT did not washout the Bt droplets on the leaves as the larval population kept decreasing until 28 DAT.

Heavy rainfall was recorded at 4 DAT (74.8 mm) during the second round of aerial spraying. The second highest rainfall was recorded on 15 DAT with 48.9 mm. Unlike the first drone spraying, heavier rainfalls (>40 mm) occurred twice in the second drone spraying operation (*Figure 6*). Besides at 4 and 15 DAT, other days from 0 to 28 DAT in the second round of drone spraying operation recorded an average rainfall ranging from 0.6-16.6 mm. However, similar to the first drone spraying operation, the rainfalls did not significantly affect the bagworm mortality with the reduction of larval population recorded at 28 DAT had exceeded 90%. Based on the results in the first and second round of drone sprayings, average rainfalls (20-40 mm) and heavy rainfall (>40 mm) did not cause significant Bt products washout as the larval population kept plummeting until 28 DAT.



Note: Number of aerial sprays was based on bagworm's population generation; spraying was conducted on 4 July 2017 as indicated by star*. The total sprayed area was 5.56 ha.

Figure 5. Average larvae and pupae population of M. plana *after the first round of drone spraying at smallholding area in Banting, Selangor, Malaysia.*



Note: Number of aerial sprays was based on bagworm's population generation; spraying was conducted on 27-28 August 2017 as indicated by star*. The total sprayed area was 5.56 ha.

Figure 6. Average larvae and pupae population of M. plana *after the second round of drone spraying at smallholding area in Banting, Selangor, Malaysia.*

Post-census After the Drone Spraying

The condition of the smallholding areas was reassessed in December 2019 which was more than two years after the drone sprayings were carried out in July and August 2017. The oil palms conditions have significantly recovered and appeared healthier compared to the conditions recorded in July 2017. The scorched-like area, which was recorded in 2017, has completely changed to a lush green area (*Figure 7*). Besides aerial monitoring using drones, the monitoring was also carried out through ground inspection. Based on the census data, the population of bagworm larvae in the area was below the economic threshold level (<10 LPF). Hence, control is not required. Observation from the ground reconfirmed the aerial view finding where the oil palms looked healthy and had fully recovered from the previous outbreak (*Figure 8*).

DISCUSSION

In this study, we have demonstrated the effectiveness of drone aerial spraying using Bt for the control of bagworms at a severely, multi-stages bagworm infested area. A notable improvement in coverage and control was observed during aerial spraying using drones compared to ground spraying. In 2013 until 2014, series of aerial sprayings were conducted to control bagworms at an oil palm plantation in Slim River, Perak using Aircraft Grumman Ag Cat model (Kamarudin *et al*., 2017). A lot of time was spent allocating and repositioning the aircraft at the end of each pass and transporting the aircraft from the loading site to the field. These time-consuming activities have been reduced when aerial spraying was conducted using drones. One of the critical problems for aerial spraying using aircraft is the requirement of a dedicated runway for take-off

(a) (b)

Figure 7. Severe infestation caused by M. plana *before treatment in 2017 and two years after the treatment in 2019. (a) Before treatment in July 2017, and (b) after treatment in December 2019.*

(a) (b)

Figure 8. Ground observation on the recovery of the oil palm before and after the Bt drone spraying. (a) Before treatment in July 2017, and (b) after treatment in December 2019.

and landing. It has been a struggle searching for the appropriate runway for the aircraft since the availability of airstrips in Malaysia for agricultural use is very lacking. The issue was somewhat resolved, by using drones as it can take off almost anywhere within the plantation without the time-consuming search for a runway. It was found that drones are very beneficial for the rapid control of bagworms, especially in discreet hotspot areas. Compared to aircrafts, drones are highly efficient, low cost and environment friendly. The first-ever drone spraying conducted on oil palm to control bagworms depicted in this study had proved to be efficient in reducing the pest population. The right strategy to implement the drone spraying needs to be carefully planned according to the bagworm life stages.

Close monitoring of bagworm census, precise timing and follow-up aerial spraying were crucial strategies for controlling the multi-stage bagworm outbreak (Kamarudin *et al*., 2017). The average life cycle for *M. plana* from egg to adult takes about 103.5 days (Kok *et al*., 2011.). Hence, in strategising for the right control plan, the census data plays the utmost importance to determine when the treatment should begin. Areas with a multi-stage infestation may require follow-up treatments depending on the severity of infestation. In the first drone spraying, the number of larvae was successfully reduced, however, at 28 DAT, the high population of pupae had necessitate a follow-up treatment in the area. The pupa represents an inactive stage or resting mode for the bagworm, of which they will be able to ingest the delta-endotoxin from Bt (Mazmira *et al*., 2011). Thus, any control treatment carried out during the pupae stage will be ineffective and wasted. The pupal population that survived the drone spraying in the first round will eventually emerge as a new bagworm generation, and this explains the increase of larval population during the 0 DAT of the second round of drone spraying. With precise strategy and right implementations, the bagworm population was successfully reduced below the economic threshold level after the second aerial spray. The results thus, encourage the usage of drones as an essential tool for controlling at bagworm outbreak. Without proper treatment and the right strategy to contain the bagworm infestation, its population will gradually increase and over time will severely damage oil palm trees. A similar observation was recorded in the control plot, where the bagworm population would not simply vanish due to environmental factors. The population will keep increasing and will affect not only the area but also the neighbouring plantations. Thus, to ensure the bagworm population is maintained below the economic injury threshold level, close monitoring and consistent census are deemed very necessary. Leaving an infected area without control would lead to an outbreak, where consequently, significant effort and high operation costs will be required to control the pest.

Besides the right timing and strategy, the droplet deposits from the drone are also crucial in determining the success of the spraying. Droplet distribution will reflect the actual deposition level of the sprayed droplets (Guo *et al*., 2019). The flight velocity and the spraying height in this study was found to be appropriate for efficient control of bagworms in oil palm plantations. The droplets from the drone were distributed evenly, which enabled comprehensive coverage on the oil palm fronds for efficient control. This study agrees with the report by Guo *et al*. (2019), where they found that the flight height and velocity remarkably influenced the average deposition number of droplets. One of the factors that need to be further investigated is the effect of the vortex on droplet deposition during the drone spraying. Study on droplet distribution needs to be undertaken since the consistent application of the best-suited droplet size will contribute to better penetration of the crop canopy, decrease drift potential and evaporation, and add greater adherence of Bt to the leaves (Miranda *et al*., 2017). In this study, it was also found that the bagworm population was not significantly affected by rainfall. A series of rainfall exceeding 40 mm did not seem to cause a washout of Bt properties. Other studies have also shown that the bagworm populations had not correlated with weather changes (Ahya Mahadi *et al*., 2012; Cheong *et al*., 2010; Ho *et al*., 2011).

The success of the drone spraying to control the bagworm population under the threshold level in the oil palm smallholding need to be adequately maintained. Bagworms can spread rapidly without proper control and monitoring. Bagworms are a recurring problem in oil palm plantations, and several factors have been identified as causes for the outbreaks. Factors such as strong wind, vehicles, animal and humans are reported as the spreading vector of bagworms (Cheong and Tey, 2012). The bagworm population in the area needs to remain under the threshold level to avoid the risk of the recurring infestation since there are many cases where bagworm outbreaks often recur even when aerial spraying has been successfully conducted (Norman and Mazmira, 2019). Thus, the introduction of other biological control agents in the area holds significant importance. The planting of beneficial plants attracts beneficial insects which synergises with Bt aerial spraying to degrade the bagworm outbreak (Mazmira *et al*., 2011). Beneficial insects such as predators and parasitoids were found to be associated with the mortality of bagworms and naturally controlling the bagworm population in oil palm plantation (Basri, 1993; Cheong *et al*., 2010; Norman and Arshad, 2016). Planters must, therefore,

seriously consider planting these beneficial plants like *Cassia cobanensis*, which provide honey from the leaf nodes as well as from the flowers (Norman *et al*., 2019). The researchers of this current study have advised the smallholders to initiate the planting of beneficial plants since the first drone aerial spraying was carried out in July 2017. The smallholders have collaborated well in maintaining the beneficial plants in their plantations, and the effects has been commendable. Synergism effects between the drone spraying using Bt and planting of beneficial plants have enabled the smallholders to avoid recurring infestations in the plantations and have successfully maintained the bagworm population under the economic threshold level even after two years since the drone spraying was first carried out. Additionally, the Bt aerial spray coupled with beneficial plants in the bagworm outbreak areas has led to a more efficient integrated pest management (IPM) which favourably necessitates novel drone applications.

CONCLUSION

In this study, the authors have documented the application of drone spraying of Bt based biopesticides for the control of bagworm outbreaks in oil palms. The efficacy of the drone spraying in reducing the bagworm outbreak under the economic threshold level at a severely infested area with multi-stages of bagworms after just two rounds of drone spraying significantly establishes a new tool for the IPM system. Application of biopesticides for bagworm control is frequently needed at a specific time and location for highly accurate site-specific management. The drone spraying is best suited for this purpose since the operation is undemanding with easy accessibility compared to agricultural aircraft. There is no requirement for a dedicated runway for drones and the logistic aspect of the operation are less consuming. With new applications being developed rapidly across many industries, including agriculture, there may be a sharp uptake of drone technology in the near future, especially in the oil palm industry. Drones are facilitating work in the agricultural field, especially in aerial spraying by reducing time and workforce. With the long term IPM strategy such as the planting of beneficial plants, the bagworm population can be maintained under the threshold for many years after completion of a successful drone spraying operation.

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REFERENCES

Ahmad, M N; Ali, S R A; Masri, M M M and Asmawi, M Z (2015). Effect of *Bacillus thuringiensis* based-products on rats. *J. Oil Palm Res., 27(1)*: 30-38.

Ahmad, M N; Ali, S R A; Masri, M M M and Wahid, M B (2012). Effect of Bt products, Lepcon-1, Bafog-1 (S) and Ecobac-1 (EC), against the oil palm pollinating weevil, *Elaeidobius kamerunicus* and beneficial insects associated with *Cassia cobanensis*. *J. Oil Palm Res., 24*: 1442-1447.

Ahmad, M N; Ali, S R A; Masri, M M M and Zolkarnain, N (2014). Lepcon-1, Bafog-1 (S) and Ecobac-1 (EC), *Bacillus thuringiensis* based-products are not toxic against freshwater fish, *Tilapia nilotica*. *J. Oil Palm Res., 26(4)*: 317-320.

Ahya Mahadi, N; Muhamad and Azura Adam, N (2012). Relationship between bagworm *Pteroma pendula* Joannis (Lepidoptera: Psychidae) populations, parasitoids and weather parameters in oil palm plantation. *J. Agric. Sci., 4(12)*: 13-17. DOI: 10.5539/jas.v4n12p13.

Amin, R I (2018). *Pesawat ringan terhempas di Muadzam Shah.* http://www.bharian.com.my/berita/kes/2018/02/392870, accessed on 1 January 2021.

Attorney General's Chambers (2013). Declaration of dangerous pest 2013. *Federal Government Gazette*. PU(B) 468.

Basri, M W (1993). Life history, ecology and economic impact of the bagworm, *Metisa plana* Walker (Lepidoptera: Phychidae), on the oil palm. *Elaeis guineensis* Jacquin (Palmae). PhD thesis. University of Guelph.

Bernama (2017). MADA creates history with drone pesticide spray. *https://www.malaymail.com/news/malaysia/2017/04/01/mada-creates-history-with drone-pesticide-spray-says-shabery/1347549,* accessed on 1 April 2021.

Cheong, Y L and Tey, C C (2012). Understanding pest biology and behaviour for effective control of oil palm bagworms. *Planter, 88(1039)*: 699-715.

Cheong, Y L; Sajap, A S; Hafidzi, M N; Omar, D and Abood, F (2010). Outbreaks of bagworms and their natural enemies in an oil palm, *Elaeis guineensis*, plantation at Hutan Melintang, Perak, Malaysia. *J. Entomol., 7*: 141-151. DOI: 10.3923/je.2010.141.151.

European Commision (2018). Digital Transformation Motor: Drones in Agriculture. p. 6.

Giles, D K and Billing, R C (2015). Deployment and performance of a UAV for crop spraying. *Chem. Eng. Trans., 44*: 307-312.

Guo, S; Li, J; Yao, W; Zhan, Y; Li, Y and Shi, Y (2019). Distribution characteristics on droplet deposition of wind field vortex formed by multi-rotor UAV. *PLoS ONE, 14*: e0220024. DOI: 10.1371/journal.pone.0220024.

Hasber, S (2010). Evaluation of several chemical control approaches against bagworm, *Metisa plana* Walker (Lepidoptera: Psychidae) in FELDA oil palm plantations. MSc. thesis. Universiti Sains Malaysia.

Ho, C T; Yusof, I and Khoo, K C (2011). Infestation by the bagworms *Metisa plana* and *Pteroma pendula* for the period 1986-2000 in major oil palm estates managed by Golden Hope Plantation Berhad in Peninsular Malaysia. *J. Oil Palm Res., 23(2)*: 1040-1050.

Kamarudin, N; Ali, S R A; Masri, M M M; Ahmad, M N; Manan, C and Norman, K (2017). Controlling *Metisa plana* Walker (Lepidoptera: Psychidae) outbreak using *Bacillus thuringiensis* at an oil palm plantation in Slim River, Perak, Malaysia. *J. Oil Palm Res., 29*: 47-54.

Kamarulbaid, A M (2018). *Guna Dron Musnah Ulat Bungkus*. Utusan Malaysia. 26 March 2021.

Kok, C C; Eng, O K; Razak, A R and Arshad, A M (2011). Microstructure and life cycle of *Metisa plana* Walker (Lepidoptera: Psychidae). *J. Sustain. Sci. Manag., 6*: 51-59.

Mat Su, A S; Yahya, A; Mazlan, N and Ahmad Hamdani, M S (2018). Evaluation of the spraying dispersion and uniformity using drone in rice field application. MSAE Conference, Serdang, Selangor. 7-8 February 2018.

Mazmira, M; Ramlah, S; Najib, M; Norman, K; Kushairi, A and Wahid, M B (2011). Pest management of bagworm in Southern Perak by aerial spraying with *Bacillus thuringiensis*. *Oil Palm Bulletin, 63*: 24-33.

Miranda, P S; Monteiro, N; Pradeep, V; Dsouza, R and Fernandes, G H (2017). Farmer friendly drone. *Int. J. Internet of Things, 6*: 56-61. DOI: 10.5923/j.ijit.20170602.10.

Rao Mogili, U M and Deepak, B B V L (2018). Review on application of drone systems in precision agriculture. *Procedia Comput. Sci., 33*: 502-509.

MPOB (2016). *Standard Operating Procedure (SOP) Guidelines for Bagworm Control*. MPOB, Bangi. 41 pp.

Norasma, C Y N; Abu Sari, M Y; Fadzilah, M A; Ismail, M R; Omar, M; H Zulkarami, B; Hassim, Y M M and Tarmidi, Z (2018). Rice crop monitoring using multirotor UAV and RGB digital camera at early stage of growth. *IOP Conf. Ser.: Earth Environ. Sci., 169*: 012095.

Norman, K; Basri, M W; Ramle, M and Ramlah, A A S (2004). Bagworms, rhinocerous beetle and nettle caterpillars - Biology, life cycle and control on oil palms in Malaysia. *Proc. Industrial Conf. Pests and Diseases of Importance to the Oil Palm Industry.* p. 96-116.

Norman, K and Arshad, O (2016). Diversity and activity of insect natural enemies of the bagworm (Lepidoptera: Psychidae) within an oil palm plantation in Perak, Malaysia. *J. Oil Palm Res., 28*: 296-307. DOI: 10.21894/jopr.2803.06.

Norman, K and Mazmira, M M M (2019). Industry-wide efforts in circumventing the scourge of bagworm infestation in Malaysia - What have gone wrong and what should be done? *Planter, 95(1118)*: 321-333.

Norman, K; Seman, I A and Masri, M M M (2019). Prospects in sustainable control of oil palm pests and diseases through the enhancement of ecosystem services - The way forward. *J. Oil Palm Res., 31*: 381-393.

Ramlah, A A S; Najib, M A and Mazmira, M M M (2011). Method of producing microbial insecticide. Malaysian Patent Application PI2011000307.

Ramlah, A A S; Norman, K; Basri, M W; Najib, M A; Mohd Mazmira, M M and Kushairi, A (2007a). *Manual Pengurusan Bersepadu Ulat Bungkus di Ladang Sawit*. MPOB, Bangi. 15 pp.

Ramlah, A A S; Norman, K; Basri, M W; Najib, M A; Mohd Mazmira, M M and Kushairi, A (2007b). *Sistem Pengurusan Perosak Bersepadu bagi Kawalan Ulat Bungkus di Ladang Sawit.* MPOB, Bangi. 28 pp.

Ramlah, A A S and Basri, M W (1997). A local *Bacillus thuringiensis* SRBT1 with potential for controlling *Metisa plana* (Wlk). *J. Oil Palm Res., 9(1)*: 34-45.

Sundaram, K M S; de Groot, P and Sundaram, A (1987). Permethrin deposits and airborne concentrations downwind from a single swath application using a back pack mist blower. *J. Environ. Sci. Health B, 22(2)*: 171-193.

Tey, C C and Cheong, Y L (2013). Challenges in integrated pest management (IPM). *Proc. of 10th NATSEM 2013 - Confronting Management Challenges in the Oil Palm Industry*. Incorporated Society of Planters, Kuala Lumpur. p. 117-127.

Theriault, R; Salyani, M and Panneton, B (2001). Spray distribution and recovery in citrus application with a recycling sprayer. *Trans. ASAE, 44(5)*: 1083-1088.

Thomas, G (2017). Treat bagworm early, MPOB. Retrieved from https://www.thestar.com.my/opinion/letters/2017/05/30/treat-bagworms-early-mpob, accessed on 30 May 2020.

Wood, B J (2002). Pest control in Malaysia's perennial crops: A half century perspective tracking the pathway to integrated pest management. *Integr. Pest Manag. Rev.*, *7*: 173-190.

Wood, B J and Kamarudin, N (2019). Bagworm (Lepidoptera: Psychidae) infestation in the centennial of the Malaysian oil palm industry - A review of causes and control. *J. Oil Palm Res., 31*: 364-380.

Zhang, D Y; Lan, Y B; Chen, L P; Wang, X and Liang, D (2018). Current status and future trends of agricultural aerial spraying technology in China. *Trans. Chin. Soc. Agric. Machinery, 45(10)*: 53-59.

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DOI: https://doi.org/10.21894/jopr.2021.0047

APPLICATION OF TARGETED GOAT GRAZING IN OIL PALM PLANTATIONS: ASSESSMENT OF WEED PREFERENCE, SPATIAL USE OF GRAZING AREA AND LIVE WEIGHT CHANGE

FRISCO NOBILLY[1,2]*; THOMAS MANLIT RAYMOND MAXWELL[3]; MUHAMMAD SYAFIQ YAHYA[4] and BADRUL AZHAR[4,5]

ABSTRACT

Targeted goat grazing is a promising tool to control competing weeds in crop plantation systems without causing adverse effects on the environment. Here, we investigated the effectiveness of targeted grazing for weed control involving 11 Katjang crossbreed goats in a mature oil palm plantation. We assessed the animal behaviour and management aspects including weed preference, spatial use of grazing area, and body weight change. Asystasia gangetica *was the most preferred weed species, followed by* Clidemia hirta. *Time spent grazing on* A. gangetica *(45.83-282.91 s) and* C. hirta *(10.04-49.82 s) by the female goats were different between grazing days (*p*<0.05). Spatial use between edge and interior areas of grazing plots were not different (*p*=0.718), meaning goats grazed evenly throughout the grazing plots. Our results revealed that goats fed evenly on the diverse weed community throughout the grazing plots and maintained similar body weight (*p*=0.488) before and after grazing. Livestock integration with oil palm agriculture in the manner of targeted grazing should be promoted as a part of integrated pest management for reducing weeds. Targeted grazing might be the solution for environmentally sound weed management in sustainable oil palm plantations.*

Keywords: biological control, integrated pest management, livestock integration, sustainable palm oil.

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INTRODUCTION

Sustainable palm oil production demands producers to improve current agricultural practices, with the intention of compatibility with environmental protection and worker health (Abdul Majid, 2021; Azhar *et al*., 2017; Saadun *et al*., 2018). Palm oil certification schemes such as the Roundtable on Sustainable Palm Oil (RSPO) and Malaysian Sustainable Palm Oil (MSPO) require producers to implement the use of biological control methods in palm oil production (Kalidas, 2012). For instance, biological control such as cover crop management has shown to be effective in reducing weeds and has the potential to be included in sustainably managed oil palm plantations to reduce the use of commercial herbicides (Baumgartner *et al*., 2008; Gago *et al*., 2007; Samedani *et al*., 2015; Tohiran *et al*., 2019a; 2019b). In Malaysia, leguminous cover crops species commonly used are *Pueraria phaseloides* (synonym for *Pueraria javanica*), *Centrosema pubescens*, *Calopogonium mucunoides*, *C. caeruleum* and of late, *Mucuna bracteata* as they reduce the growth and cover of weed species (Mathews and Saw, 2007). This improvement of agricultural practice is inevitable

[1] Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

[2] Laboratoire d'Excellence (LabEx), Sustainable Tropical Agriculture and Food Systems, UPM-Agropolis International Offshore Office, F-34394 Montpellier CEDEX 5, France.

[3] Department of Agricultural Sciences, Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln 7647, Christchurch, New Zealand.

[4] Department of Forestry Science and Biodiversity, Faculty of Forestry and Environment, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

[5] Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

* *Corresponding author e-mail: frisco@upm.edu.my*

due to the increasing market demand for certified palm oil-based products globally (CPET, 2015). It is forecasted that the scale of oil palm plantation land use will continue to expand in Southeast Asia, Western Africa and South America (Vijay *et al*., 2016). To date, there is a lack of empirical evidence on biological control as an effective alternative to manage weeds in oil palm plantations.

Weed species such as *Chromolaena odorata* and *Asystasia gangetica* are mainly controlled by chemical herbicides in oil palm plantations because they compete with the palm oil crop for water and nutrients, reducing crop health and productivity (Ali *et al*., 2021; Nchanji *et al*., 2016; Satriawan *et al*., 2021; Sidik *et al*., 2018). Chemical herbicides such as paraquat (600 or 800 g ha^{-1}), gluphosinate-ammonium (200 g ha^{-1}) and glyphosate (400 g ha^{-1}) are commonly used to control weeds in oil palm plantations (Dilipkumar *et al*., 2017; Mohamad *et al*., 2010; Wibawa *et al*., 2010). Major palm oil producing countries such as Malaysia have banned the use of herbicides such as paraquat (Wibawa *et al*., 2007), deemed hazardous to human health and the environment (Ferramosca *et al*., 2021; Pochron *et al*., 2021; Van Bruggen *et al*., 2018; Weisenburger, 1993; Zaller *et al*., 2021). An alternative to using the remaining permitted chemical herbicides for controlling weeds in oil palm plantations is the application of targeted grazing with livestock animals (Tohiran *et al*., 2017; 2019a; 2019b).

Targeted grazing is grazing by ruminant livestock for a set duration and intensity during a specified stage in the growing season to achieve specific vegetation management goals (Frost and Launchbaugh, 2003; Launchbaugh *et al*., 2006; Walker, 1994). It is an under-exploited tool that is fast proving very potent for weed control in expansive rangeland scenarios (Bailey *et al*., 2019; Frost and Launchbaugh, 2003). Using targeted grazing effectively demands site-specific knowledge of plant growth, animal nutrition, grazing behaviour, ecosystem function and public relations (Macon, 2014). Compared with herbicide application, targeted grazing can be inexpensive, with very light targeted grazing by sheep reducing weeds and increasing desired plant species (Bailey *et al*., 2019; Rinella and Hileman, 2009).

Goat grazing can be an important tool for biodiversity conservation in agroecosystems if managed appropriately (Azhar *et al*., 2021; García *et al*., 2012). The integration of rotationally grazed livestock in oil palm plantations has been promoted in order to manage weeds, supplement crop growth with organic manure, and improve farmland biodiversity (Azhar *et al*., 2013; Jambari *et al*., 2012; Tohiran *et al*., 2017). In established oil palm plantations, noxious weeds such as *Asystasia gangetica*, *Clidemia hirta, Centhotheca lappacea* and *Cyrtococum accrescens* strongly compete with oil palm trees for nutrients, moisture and sunlight and eventually cause yield depression (Azahari *et al*., 2004). At establishment and early growth stages of oil palm trees, weeds compete for resources, negatively affecting tree growth and yield and obstructing routine estate practices such as harvesting (Azahari *et al*., 2004; Rosli *et al*., 2010). Eradication of a very dense stand of *A. gangetica* in an oil palm plantation resulted in a 12% increase in FFB production (Kustyanti and Horne, 1991). Although the most common cost-effective practice to remove oil palm plantation weeds is herbicide application (Wibawa *et al*., 2010), this method is becoming unfavourable and increasing unpopular with the public due to the toxic and hazardous effects (Farooq *et al*., 2011). Biological weed control using grazing animals offers an economically cheaper alternative compared to herbicides in crop-livestock integration systems (Sánchez, 1995; Tohiran *et al*., 2014; 2017).

Feeding behaviour of goats in both open and confined areas influence their diets (Araújo *et al*., 2018; Goetsch *et al.,* 2010). Previous research has reported that feeding behaviour can be influenced by genotype, environmental conditions (site), differences in vegetation conditions (quality), and preference for different plant species (Goetsch *et al*., 2010; Mphinyane *et al*., 2015). However, feeding behaviours of goats raised in the field were almost the same as those raised in a confined area (Silva *et al.,* 2013). Most goat feeding behaviours appear to be influenced by the level of concentrate in the ration (Lu *et al*., 2008) and the physical characteristics of the fibre (Lu *et al*., 2008; Zhao *et al*., 2014). Additionally, goat feeding behaviour varies differently with the body size of different genotypes (Koluman *et al*., 2016).

Animal feeds of natural grass and total mixed rations have different crude fibre content and texture which influence feeding, rumination and chewing activity of goats (Adiwinarti *et al*., 2019). The more dietary fibre intake by goats, the longer the chewing time required (Lu *et al*., 2008). Goats fed with high fibre feed needed more time to eat and had longer feeding bouts, causing lower feed intake than goats fed low fibre feed (Adiwinarti *et al*., 2019). In comparison, goats fed with total mixed rations require more drinking water than goats fed on natural grass (Adiwinarti *et al*., 2019).

In a grassland environment, grazing has a profound influence on the diversity and spatial distribution of plant species (Adler *et al*., 2001). Selective grazing of particular species can lead to local extinction or colonisation of plant species, and changes in the within-community spatial organisation of vegetation (Pazos *et al*., 2007). Additionally, the heterogeneous spatial distribution of local disturbances induced by

livestock grazing, such as trampling, gap creation, or nutrient deposition, can also create spatial heterogeneity in plant species composition (Adler *et al*., 2001; Augustine and Frank, 2001). Similar approaches can be applied to crop production, especially in oil palm plantation scenarios. Understanding the spatial distribution of livestock movement around a plantation site can provide significant management tools for farm managers (Sanderson *et al*., 2010).

Our study assessed the grazing and browsing behaviour and management aspects of targeted goat grazing in an oil palm agro-ecosystem. Specific objectives of the study were: (i) to determine weed preference by local goats (Katjang crossbreed), (ii) determine spatial use of grazing plots by goats, and (iii) measure live weight of goats gain in response to targeted grazing on understory weeds. This study sheds new light on the application of grazing livestock as a potential biological weed control method in sustainable palm oil production.

MATERIALS AND METHODS

Study Site

This single livestock species grazing study was conducted at the Malaysian Palm Oil Board (MPOB) Keratong Research Station, Pahang, Malaysia (N 02º47′56.1″ E 102º55′37.6″) between July and August 2016. The research station is located in a 700 ha oil palm plantation, with a planting density of 136 palms per ha. The oil palm stand was 17 years old. Weed control using chemical herbicide is sprayed three to four times per year and has been the standard practice for more than 25 years.

Experimental Design and Animals

Seven, 25.0 m × 25.0 m randomly allocated grazing plots within the 700 ha plantation were erected using portable net-type electric fences, powered by 12 volt rechargeable lead battery. Individual grazing plots were divided into three rectangular strips (8.3 m × 25.0 m) with each strip allocated for one day of grazing. The grazing plots were characterised by a flat area of nine oil palm trees. A total of 11 healthy Katjang crossbreed (*Capra hircus*) goats, comprising one buck and 10 does, aged between 12 to 24 months were used in this study. The goats were fed with commercial pellets (soybean hull) and oil palm fronds leaves before the grazing commenced. Each individual goat was ear tagged for identification. Goats were released from their shelter and herded to their allocated sub-plot at 10.00 am and then herded back to their shelter at 2.00 pm daily (approximately 4 hr of grazing daily). Each goat was supplemented with 200 g soybean hull pellets and *ad libitum* supply of chopped palm oil fronds, after returning to their shelter. Goats were allowed to graze in each grazing sub-plot for one day before moving to an adjacent plot. Each goat was first weighed prior to grazing and re-weighed every seven days using a portable digital weighing scale. The mean initial doe body weight was 17.67 kg, while the buck was 31.40 kg. Prior to commencement of the targeted grazing experiment, goats were trained to familiarise with the live electric fencing. During the study period, the grazing plot areas were not sprayed with herbicides. Out of the 11 animals used in this study, unfortunately three had died due to parasite infection.

Goat Grazing Preference for Weed Species

We used scan sampling to randomly select seven individual goats and recorded the types of weed species they selected during a 15 min browsing bout. We used a digital stopwatch to record the duration of time spent browsing a particular weed species. This 15 min duration was counted as a visual observation. In this study, we recorded a total of 147 visual observations.

Spatial Use of Grazing Plot

At 15 min intervals, we determined the specific location of each goat, either at the edge (within 5 m from the fence) or at the interior (beyond 5 m of the fence) of the grazing plot. This 15 min interval was counted as a visual observation adapted from Parsons and Dumont (2003). A total of 378 visual observations were recorded.

Data Analysis

Prior to data analyses, Shapiro-Wilk's tests were performed to determine the distribution pattern of collected data. To improve the linearity of data distribution, data was either square root or log transformed. To compare the number of weed species browsed by different individual animals and grazing days (day 1-21), we performed a balanced one-way analysis of variance (ANOVA). We used *post hoc* analysis (*i.e*., Tukey's test) to conduct multiple comparisons of different treatment (grazing day) levels. To compare the grazing time between different spatial areas (edge or interior of grazing plot), we conducted an unbalanced one-way ANOVA. To compare the body weight of does, we performed a balanced one-way ANOVA and *post hoc* analysis (*i.e*., Tukey's test). The buck was excluded from the data analyses. We performed all statistical analyses in GenStat version 15 (VSNI, Hemel Hempstead United Kingdom).

RESULTS AND DISCUSSION

Weed Preference and Control

Thirteen weed species were grazed and browsed by goats (*Table 1*). Within each grazing plot, the weed cover was completely cleared after three days of grazing (*Figure 1*). There was no significant difference in the number of browsed weed species between the seven different individual goats (df=6; variance ratio=0.75; p=0.607; *Figure 2*). This result indicates that each doe was consistent with respect to the number of weed species that it could control in oil palm plantations. However, there was a significant difference in the number of weed species browsed by goats between different days (df=20; variance ratio=2.29; p=0.003; *Figure 3*).

Based on the cumulative data of time spent grazing, *Asystasia gangetica* was the most preferred weed species during the first day at each grazing plot, followed by *Clidemia hirta* (*Table 1*). There was a significant difference in time spent grazing *A. gangetica* (df=6; variance ratio=5.86; p<0.001) and *C. hirta* (df=6; variance ratio=2.29; p=0.039) between the seven individual female goats (*Figure 3*). Similarly, time spent grazing *A. gangetica* (df=20; variance ratio=5.21; p<0.001) and *C. hirta* (df=20; variance ratio=5.28; p<0.001) by the female goats was different between grazing days (*Figure 3*). Our results show that time spent browsing on *Centhotheca lappacea* (df=20; variance ratio=5.29; p<0.001) and *Ischaemacum muticum* (df=20; variance ratio=1.95; p=0.014) was significantly different between days (*Figure 3*).

Time spent grazing *Cyrtococum accrescens* was not significantly different between the seven individual does, nor between the grazing days (*Figure 3*). There was also no significant difference in time spent grazing *C. lappacea* and *I. musticum* between the seven individual does (*Figure 3*).

TABLE 1. CHECKLIST OF WEED SPECIES GRAZED BY GOATS IN AN OIL PALM PLANTATION

Species	Family	Growth habit	Duration	Cumulative time spent on feeding by goats (s)
Asystasia gangetica	Acanthaceae	Forb/herb	Perennial	31 054
Clidemia hirta	Melastomataceae	Shrub	Perennial	7 171
Centotheca lappacea	Poaceae	Graminoid	Perennial	3 558
Cyrtococum accrescens	Poaceae	Graminoid	Annual	2 518
Ischaemum muticum	Poaceae	Graminoid	Perennial	1 410
Stenochlaena palustris	Blechnaceae	Forb/herb	Perennial	250
Chromolaena odorata	Asteraceae	Shrub	Perennial	185
Nephrolepis biserrata	Lomariopsidaceae	Forb/herb	Perennial	96
Davallia denticulata	Davalliaceae	Forb/herb	Perennial	84
Adiantum latifolium	Pteridaceae	Forb/herb	Perennial	75
Vittaria elongata	Pteridaceae	Forb/herb	Perennial	46
Paspalum dilatum	Poaceae	Graminoid	Perennial	3
Paspalum conjugatum	Poaceae	Graminoid	Perennial	1

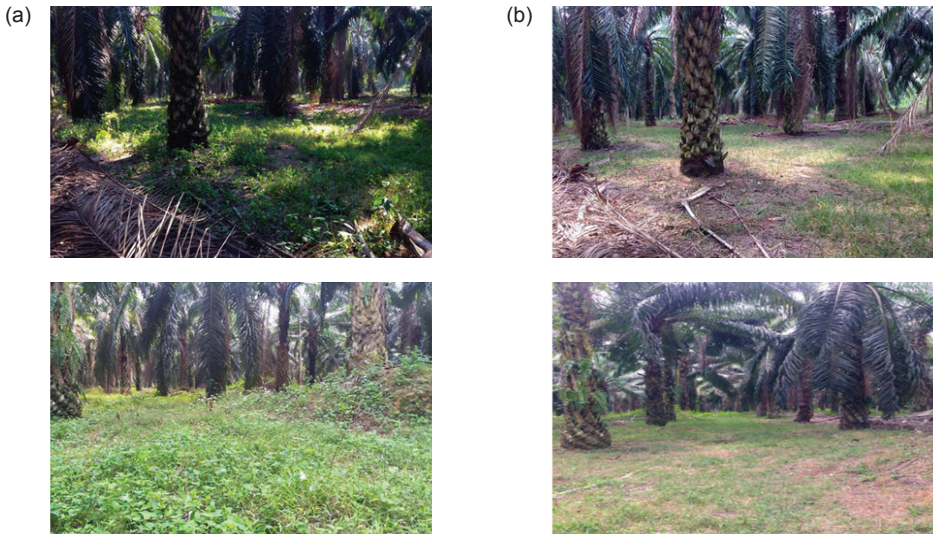

Figure 1. (a) Weed cover in oil palm plantations before, and (b) after targeted goat grazing over three days.

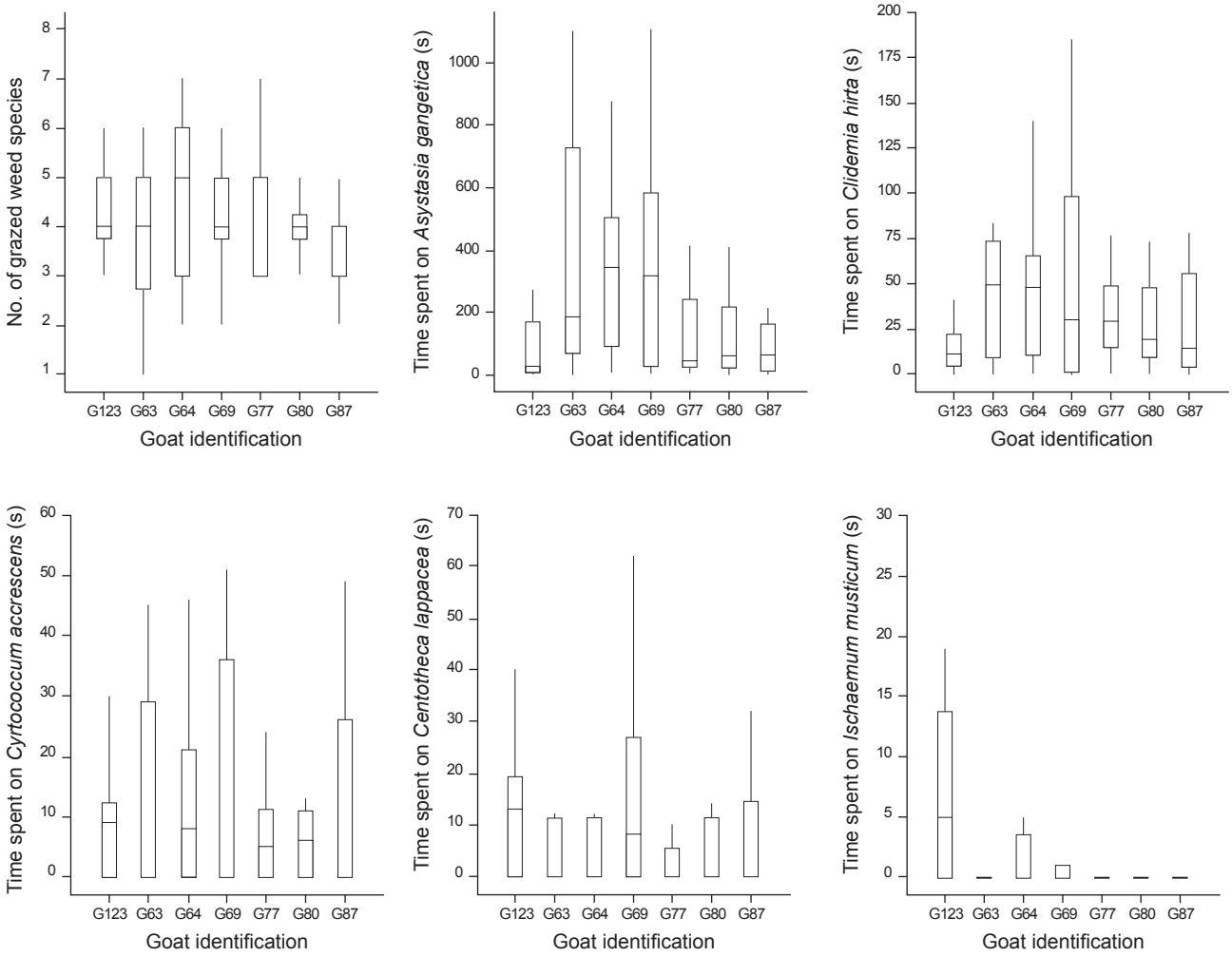

Figure 2. Boxplots of the number of weed species grazed, and top five weed species preferred by goats with respect to individual animals grazing time within grazing plots in an oil palm plantation.

Spatial Use of Grazing Plots

No significant difference in spatial use between edge and interior areas of grazing plots was observed (df=1; variance ratio=0.13; p=0.718). The goats grazed evenly throughout the grazing plots without displaying spatial bias towards the edge or interior areas. In addition, grazing days had no significant effect on spatial use (df=2; variance ratio=1.04; p=0.355).

Live Body Weight Gain

There was no significant difference in body weight, before and after grazing, among the 10 does (df=4; variance ratio=0.87; p=0.488; *Figure 4*). Our data indicate that the body weights decreased in the first week ($\bar{x}$=16.82 kg; day 7) and second week ($\bar{x}$=17.19 kg; day 14), but gradually increased in the third week ($\bar{x}$=17.83 kg; day 21) and fourth week ($\bar{x}$=18.25 kg; day 28; *Figure 4*).

Goat Preferences for Weed Species

Our data indicate Katjang crossbreed goats consume ground level weeds, but they also browse taller and woodier vegetation. As efficient browsers, goats have a unique character that distinguishes them from almost all other types of livestock. Browsing makes up approximately 60% of a goat's activity but only about 10%-15% of a cow's activity (Bull, 2000). In oil palm plantations, prevalent weeds species such as *A. gangetica*, *C. hirta* and *C. odorata* are readily available and have become the common plant species of the understory ground layer of Malaysian oil palm plantations (Wibawa *et al*., 2009). This study suggests that these three weed species have lower fibre content. Goats prefer to browse weed species with greater palatability and lower fibre content (Nampanzira *et al*., 2015), with *A. gangetica* being the most preferred species (Achonwa *et al*., 2017).

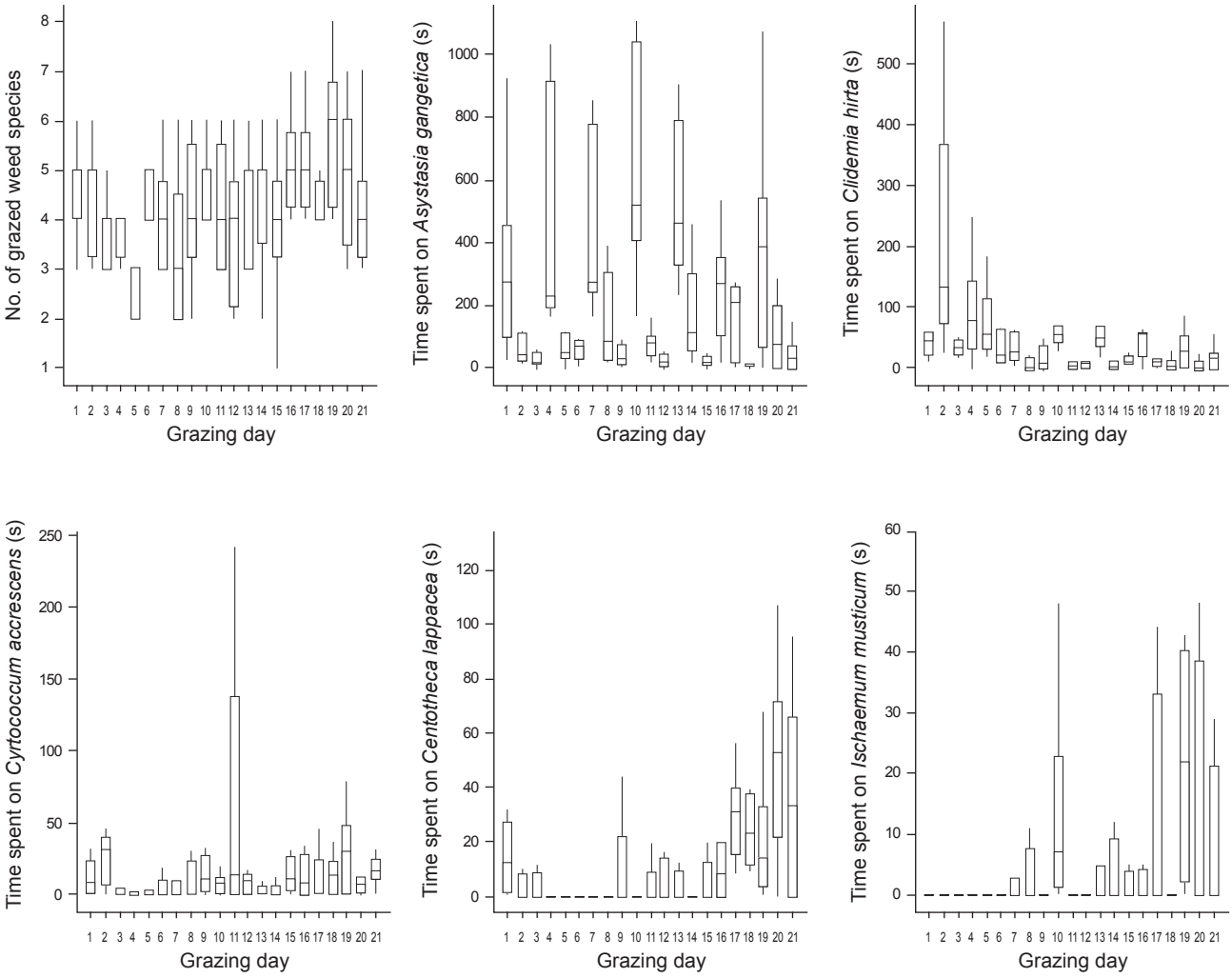

Figure 3. Boxplots of the number of weed species and top five weed species preferred by goats with respect to grazing day in an oil palm plantation.

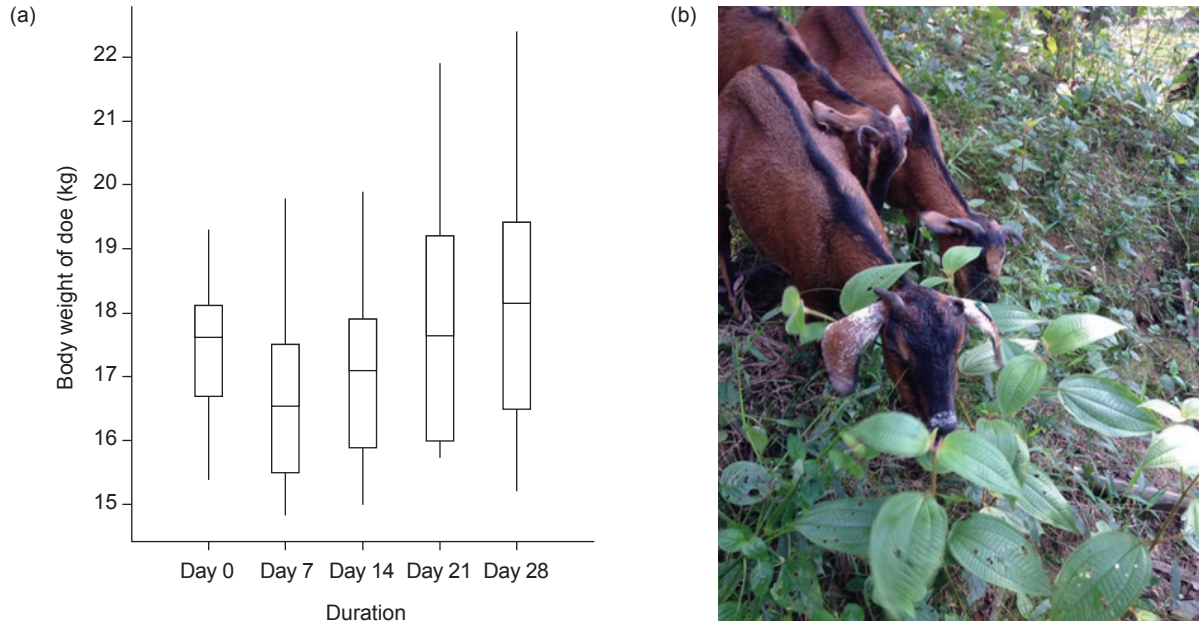

Figure 4. (a) Goat performance during the implementation of targeted grazing and (b) goats browse weeds that would be conventionally controlled by herbicides in oil palm plantations.

Understanding goat grazing behaviour in oil palm plantation areas is important knowledge for a plantation's manager if they wish to practice targeted grazing for weed control as an alternative to herbicide application. Goats and sheep have been shown to graze or browse in cyclic patterns, devouring substantial amounts of weed species during one grazing period, followed by low weed consumption in the following period (Launchbaugh *et al*., 2006). Our results indicate that goat selection was consistent across all scales which is consistent to the finding of Skarpe *et al*. (2007). Of the weed species present, goats selected to browse from relatively high trees and from trees with signs of previous browsing which is also consistent with Skarpe *et al*., 2007. Goats are known to be agile compared with cattle and sheep, frequently using a bipedal stance and climbing to gain access to vegetation of interest (Animut and Goetsch, 2008).

Goats prefer to browse several plant species that are considered weeds in typical oil palm plantation, *e.g*., *C. hirta* and *C. odorata*. In addition, goats are known for their consumption of seeds and reproductive stems, and the ability to decrease spread of some undesirable plant species (Animut and Goetsch, 2008). By using goats, control of unwanted weed species in oil palm plantations can be achieved via frequent defoliation of the undesirable species, removal of active growing points, and at high and frequent enough intensities to deplete root energy reserves. However, this may require standard or even higher stocking rates (Gabdo and Abdlatif, 2013).

The first objective of the study was to investigate weed species preferences by local Katjang crossbreed goats as a potential management practice to control weeds in the oil palm plantation. In our study, most of the weeds selected by goats were as nutritious as some common grass species available at the plantation *i.e*., *Centotheca lappacea, Cyrtococum accrescens* and *Paspalum dilatum* (Gibson, 2009). Careful planning for utilising the weeds will produce reasonable goat performance. It is reasonable to maintain and leave some weed cover for the next defoliation. Appropriate utilisation of the weeds so as not to kill them may result in underutilisation of the weeds biomass, due to goat browsing preferences.

Spatial Use of Grazing Plots

Our findings highlight that targeted goat grazing controls weed cover consistently throughout the grazing plots. It also indicates that goats are an effective biological control agent for weeds in an oil palm plantation and that portable net electric fencing is indispensable in managing the movement of goats during grazing. The stocking density (*i.e*., approximately 5 m^2 per goat per hr) is sufficient to clear the weeds in oil palm plantation. High stocking density stimulates feeding behaviours that increase utilisation of weed biomass by goats (Utsumi *et al*., 2010).

Goat Performance

The goats used in this study did not show an overall increase in body weight. The goats lost weight in the first half of the experiment and regained body weight again in the latter half of the grazing period. Our results indicate that goats can browse on weeds in oil palm plantations, without a severe decrease in live weight gain. Goat's live weight gain may be improved by adjusting stocking rates and the intensity and frequency of defoliation of the target weed species. The information gained here from the first objective, that goats show higher browsing preferences for the most prevalent weed species, which can help to refine future studies on managing goat browsing of weeds to achieve live weight gain targets as well as desired weed control.

Common weed plants in oil palm plantations can provide high forage value for livestock (Tohiran *et al*., 2014; 2017; 2019a; 2019b). The leaves and parts of woody plants browsed by the goats in oil palm plantations can be highly nutritious (Tohiran *et al*., 2014). Animal body weight indicates the type and amount of vegetation present (Launchbaugh *et al*., 2006). Hence, goat performance depends on stocking density allowing for selection of most nutritious weeds, grazing time, and on the level of supplementary stall feeding.

Future of Targeted Grazing for Weed Management

Targeted goat grazing practice is an environmentally sound method for controlling weeds in mature (*i.e*., more than five years) oil palm plantations. Moreover, this practice of using livestock grazing or browsing for short periods at high intensity can reduce the presence of pest plants (Launchbaugh *et al*., 2006). This agricultural practice, which integrates commodity crop and livestock production, inclines toward integrated pest management and reduced synthetic herbicide inputs. Our study revealed that goats fed on a diverse weed community, grazed weeds evenly throughout the grazing plots, and overall maintained similar body weight over the course of grazing in the plantation. This can address a number of the criteria for sustainability defined by the MSPO and other palm oil certification schemes (*e.g*., the RSPO). These include safe (or reduced) use of agrochemicals to protect human health and the environment, and application of integrated pest management systems to control weeds. In addition, oil palm-livestock integration practice in

the manner of targeted grazing is compatible with the United Nations Sustainable Development Goals (SDGs).

Targeted grazing can be used to accomplish vegetation management goals (*e.g.*, lowered costs for management weed control (Frost *et al*., 2012). As such, targeted grazing is being rediscovered as a viable and chemical-free tool to control weeds in Southeast Asia (Tohiran *et al.*, 2014; 2019b) which can be practically applied for sustainable palm oil production and enhanced biodiversity conservation in conventional oil palm agriculture (Tohiran *et al*., 2017; 2019a; 2019b). Further experiments are required to optimise the application of targeted grazing in oil palm plantations. These may include studies on vegetation structure and composition, soil compaction, multiple stocking density and grazer species/breeds, organic carbon level in soil, and wider ecological impacts (Launchbaugh *et al*., 2006).

CONCLUSION

The application of targeted goat grazing to control weeds in oil palm plantations can be agriculturally effective. Our study shows that targetted grazing with livestock can be as effective as herbices application in palm oil plantation (Tohiran *et al*., 2017). We recommend the floristic composition of weeds and ground coverage area to be measured in the future, in order to estimate the stocking density of the animal for weed control application. Livestock integration with oil palm agriculture in the manner of targeted grazing should be promoted as a part of integrated pest management to manage weeds.

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REFERENCES

Abdul Majid, N; Ramli, Z; Md Sum, S and Awang, A H (2021). Sustainable palm oil certification scheme frameworks and impacts: A systematic literature review. *Sustainability*, *13(6)*: 3263.

Achonwa, C C; Ogbuewu, I P; Ogundu, E C; Kubkomawa, I H; Uchegbu, M C and Okoli, I C (2017). Browse abundance and the methodologies for their selection as candidate feed resources in Nigeria: A review. *Nat. Sci., 15(5)*: 82-94.

Adiwinarti, R; Lestari, C M S and Purnomoadi, A (2019). Feeding behaviour and drinking activities of Kacang goat fed high and low fibre diets. *IOP Conf. Ser.: Earth Environ. Sci., 247(1)*: 012020.

Adler, P; Raff, D and Lauenroth, W (2001). The effect of grazing on the spatial heterogeneity of vegetation. *Oecologia, 128*: 465-479.

Ali, N B M; Karim, M F A; Saharizan, N; Adnan, N S; Mazri, N H; Fikri, N A; Amaludin, N A and Zakaria, R (2021). Weeds diversity in oil palm plantation at Segamat, Johor. *IOP Conf. Ser.: Earth Environ. Sci., 756(1)*: 012034.

Animut, G and Goetsch, A L (2008). Co-grazing of sheep and goats: Benefits and constraints. *Small Rumin. Res., 77(2)*: 127-145.

Araújo, R A; Neiva, J N M; Pompeu, R C F F; Cândido, M J D; Rogério, M C P; Lucas, R C; Maranhão, S R; Fontinele, R G and Egito, A S (2018). Feeding behavior and physiological parameters of rearing goats fed diets containing detoxified castor cake. *Semin. Ciênc. Agrár., 39(5)*: 2247-2260.

Augustine, D J and Frank, D A (2001). Effects of migratory grazers on spatial heterogeneity of soil nitrogen properties in a grassland ecosystem. *Ecology, 82*: 3149-3162.

Azhar, B; Sapari, M; Zulkifly, S; Suhailan, W M and Sajap, A S (2013). Protecting biodiversity outside natural forests: Environmental friendly oil palm plantations as an off-reserve strategy in Peninsular Malaysia. *Pertanika J. Trop. Agric. Sci., 36(S)*: 231-246.

Azhar, B; Saadun, N; Prideaux, M and Lindenmayer, D B (2017). The global palm oil sector must change to save biodiversity and improve food security in the tropics. *J. Environ. Manage., 203*: 457-466.

Azhar, B; Nobilly, F; Lechner, A M; Tohiran, K A; Maxwell, T M R; Zulkifli, R; Kamel, M F and Oon, A (2021). Mitigating the risks of indirect land use change (ILUC) related deforestation from industrial palm oil expansion by sharing land access with displaced crop and cattle farmers. *Land Use Policy, 107*: 105498.

Azahari, M; Samingin, I and Seman, I A (2004). Weed management. *Oil Palm Cultivation in Malaysia* (Ghani, E A; Zakaria, Z Z and Wahid, M B eds.). MPOB, Bangi.

Bailey, D W; Mosley, J C; Estell, R E; Cibils, A F; Horney, M; Hendrickson, J R; Walker, J W; Launchbaugh, K L and Burritt, E A (2019). Synthesis paper: Targeted livestock grazing: Prescription for healthy rangelands. *Rangel. Ecol. Manag*., *72(6)*: 865-877.

Baumgartner, K; Steenwerth, K L and Veilleux, L (2008). Cover-crop systems affect weed communities in a California vineyard. *Weed Sci., 56*: 596-605.

Bull, B (2000). Using goats for vegetation management. Nobel news and views. https://www.noble.org/news/publications/ag-news-and-views/2000/september/using-goats-for-vegetation-management, accessed on 12 December 2018.

CPET (2015). UK consumption of sustainable palm oil. CPET Annual review of UK palm oil consumption 2015. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/ attachment_data/file/477236/cpet-annual-review-consumption-palm-oil-2015, accessed on 12 December 2018.

Dilipkumar, M; Chuah, T S; Goh, S S and Sahid, I (2017). Weed management issues, challenges and opportunities in Malaysia. *Crop Prot., 134*: 104347.

Farooq, M; Jabran, K; Cheema, Z A; Wahid, A and Siddique, K H M (2011). The role of allelopathy in agricultural pest management. *Pest. Manag. Sci., 67*: 494-506.

Ferramosca, A; Lorenzetti, S; Di Giacomo, M; Murrieri, F; Coppola, L and Zara, V (2021). Herbicides glyphosate and glufosinate ammonium negatively affect human sperm mitochondria respiration efficiency. *Reprod. Toxicol., 99*: 48-55.

Frost, R A and Launchbaugh, K L (2003). Prescription grazing for rangeland weed management: A new look at an old tool. *Rangelands, 25*: 43-47.

Frost, R; Walker, J; Madsen, C; Holes, R; Lehfeldt, J; Cunningham, J; Voth, K; Welling, B; Davis, T Z; Bradford, D; Malot, J and Sullivan, J (2012). Targeted grazing: Applying the research to the land. *Rangelands, 34*: 2-10.

Gabdo, B and Abdlatif, I B (2013). Analysis of the benefits of livestock to oil palm in an integrated system: Evidence from selected districts in Johor, Malaysia. *J. Agric. Sci., 5*: 47-55.

Gago, P; Cabaleiro, C and Garcia, J (2007). Preliminary study of the effect of soil management systems on the adventitious flora of a vineyard in North-Western Spain. *Crop Prot., 26*: 584-591.

García, R R; Celaya, R; García, U and Osoro, K (2012). Goat grazing, its interactions with other herbivores and biodiversity conservation issues. *Small Ruminant Res., 107(2)*: 49-64.

Gibson, D J (2009). *Grasses and Grassland Ecology*. Oxford University Press Inc. New York.

Goetsch, A L; Gipson, T A; Askar, A R and Puchala, R (2010). Invited review: Feeding behavior of goats'. *J. Anim. Sci., 88(1)*: 361-373.

Jambari, A; Azhar, B; Ibrahim, N L; Jamian, S; Hussin, A; Puan, C L; Mohd Noor, H; Yusof, E and Zakaria, M (2012). Avian biodiversity and conservation in Malaysian oil palm production areas. *J. Oil Palm Res., 24*: 1277-1286.

Kalidas, P (2012). Pest problems of oil palm and management strategies for sustainability. *Agrotechnol., S11.* DOI: 10.4172/2168-9881.S11-002.

Koluman, N; Boga, M; Silanikove, N and Gorgulu, M (2016). Performance and eating behaviour of crossbred goats in Mediterranean climate of Turkey. *Rev. Bras. de Zootec., 45(12)*: 768-772. DOI: 10.1590/s1806-92902016001200006.

Kustyanti, T and Horne, P (1991). The effect of *Asystasia* on the growth of young rubber in polybags. http://pdf.usaid.gov/pdf_docs/PDABG454.pdf., accessed on 12 January 2017.

Launchbaugh, K; Walker, J and Daines, R L (2006). Targeted grazing: A natural approach to vegetation management and landscape enhancement. American Sheep Industry Association, Englewood, Colorado, USA. 199 pp.

Lu, C D; Kawas, J R and Mahgoub, O G (2008). Review: Recent advancements in fibre digestion and utilization in goats. *Trop. Subtrop. Agroecosystems, 9*: 65-72.

Macon, D (2014). The art and science of targeted grazing - A producer's perspective. *Rangelands, 36(5)*: 31-35.

Mathews, J and Saw, E K (2007). IOI's experiences with establishing *Mucuna bracteata* on soil derived. *Mucuna bracteata* (Goh, K J and Chiu, S B eds.). Agricultural Crop Trust (ACT), Petaling Jaya. Malaysia.

Mohamad, R; Wibawa, W; Mohayidin, M G; Puteh, A; Juraimi, A S; Awang, Y and Mohd Lassim, M (2010). Management of mixed weeds in young oil-palm plantation with selected broad-spectrum herbicides. *Pertanika J. Trop. Agric. Sci., 33(2)*: 193-203.

Mphinyane, W N; Tacheba, G and Makore, J (2015). Seasonal diet preference of cattle, sheep and goats grazing on the communal grazing rangeland in the Central District of Botswana. *Afr. J. Agric. Res., 10(29)*: 2791-2803.

Nampanzira, D K; Kabasa, J D; Nalule, S A; Nakalembe, I and Tabuti, J R S (2015). Characterization of the goat feeding system among rural small holder farmers in the semi-arid regions of Uganda. *SpringerPlus, 4(1)*: 188.

Nchanji, Y K; Nkongho, R N; Mala, W A and Levang, P (2016). Efficacy of oil palm intercropping by smallholders. Case study in South-West Cameroon. *Agroforest. Syst., 90*: 509-519.

Parsons, A J and Dumont, B (2003). Spatial heterogeneity and grazing processes. *Anim. Res., 52*: 161-179.

Pazos, G E; Bisigato, A J and Bertiller, M B (2007). Abundance and spatial patterning of coexisting perennial grasses in grazed shrublands of the Patagonian Monte. *J. Arid Environ., 70*: 316-328.

Pochron, S T; Mirza, A; Mezic, M; Chung, E; Ezedum, Z; Geraci, G; Mari, J; Meiselbach, C; Shamberger, O; Smith, R; Tucker, W J and Zafar, S (2021). Earthworms *Eisenia fetida* recover from Roundup exposure. *Appl. Soil Ecol., 158*: 103793.

Rinella, M J and Hileman, B J (2009). Efficacy of prescribed grazing depends on timing intensity and frequency. *J. Appl. Ecol., 46(4)*: 796-803.

Rosli, B M; Wibawa, W; Mohayidin, M G; Puteh, A B; Juraimi, A S; Awang, Y and Lassim, M B M (2010). Management of mixed weeds in young oil-palm plantation with selected broad-spectrum herbicides. *Pertanika J. Trop. Agr. Sci., 33*: 193-203.

Saadun, N; Lim, E A L; Esa, S M; Ngu, F; Awang, F; Gimin, A; Johari, I H; Firdaus, M A; Wagimin, N I and Azhar, B (2018). Socio-ecological perspectives of engaging smallholders in environmental-friendly palm oil certification schemes. *Land Use Policy, 72*: 333-340.

Samedani, B; Juraimi, A S; Rafii, M Y; Awadz, S S; Anwar, M P and Anuar, A R (2015). Effect of cover crops on weed suppression in oil palm plantation. *Int. J. Agric. Biol., 17*: 251-260.

Sánchez, M (1995). Integration of livestock with perennial crops. *World Animal Review, 82(1)*: 50-57.

Sanderson, M A; Feldmann, C; Schmidt, J; Hermann, A and Taube, F (2010). Spatial distribution of livestock concentration areas and soil nutrients in pastures. *J. Soil Water Conserv., 65*: 180-189.

Satriawan, H; Fuady, Z and Fitri, R (2021). Physical and chemical properties of oil palm land which overgrown with weeds at different plant age. *IOP Conf. Ser.: Earth Environ. Sci., 749(1)*: 012014.

Sidik, S; Purba, E and Yakub, E N (2018). Population dynamics of weeds in oil palm (*Elaeis guineensis* Jacq.) circle weeding area affected by herbicide application. *IOP Conf. Ser.: Earth Environ. Sci., 122(1)*: 012069.

Silva, C M; Furtado, D A; Medeiros, A N; Saraiva, E P; Guimaraes, M C C; Tota, L C A and Lopes, K B P (2013). Ethogram of three genetic groups of goats confined using monitoring video images. *Rev. Etol., 12(1-2)*: 1-11.

Skarpe, C; Jansson, I; Seljeli, L; Bergström, R and Røskaft, E (2007). Browsing by goats on three spatial scales in a semi-arid savanna. *J. Arid Environ., 68(3)*: 480-491.

Tohiran, K A; Omar, R Z; Khasim, N; Rodi, M Z; Basri, N K and Omar, W (2014). Transforming oil palm plantation for forage and livestock integration. *Oil Palm Bulletin, 69*: 1-4.

Tohiran, K A; Nobilly, F; Zulkifli, R; Maxwell, T; Moslim, R and Azhar, B (2017). Targeted cattle grazing as an alternative to herbicides for controlling weeds in bird-friendly oil palm plantations. *Agron. Sustain. Dev., 37*: 62.

Tohiran, K A; Nobilly, F; Maxwell, T; Puan, C L; Zakaria, M; Zulkifli, R; Ashton-Butt, A and Azhar, B (2019a). Cattle grazing benefits farmland bird community composition in oil palm plantations. *Ornithol. Sci., 18(1)*: 81-94.

Tohiran, K A; Nobilly, F; Zulkifli, R; Ashton-Butt, A and Azhar, B (2019b). Cattle-grazing in oil palm plantations sustainably controls understory vegetation. *Agric. Ecosyst. Environ., 278*: 54-60.

Utsumi, S A; Cibils, A F; Estell, R E; Baker, T T and Walker, J W (2010). One-seed juniper sapling use by goats in relation to stocking density and mixed grazing with sheep. *Rangel. Ecol. Manag., 63(3)*: 373-386.

Van Bruggen, A H C; He, M M; Shin, K; Mai, V; Jeong, K C; Finckh, M R and Morris, J G Jr (2018). Environmental and health effects of the herbicide glyphosate. *Sci. Total Environ., 616-617*: 255-268.

Vijay, V; Pimm, S L; Jenkins, C N and Smith, S J (2016). The impacts of oil palm on recent deforestation and biodiversity loss. *PLoS ONE, 11(7)*: e0159668.

Walker, J W (1994). Multispecies grazing: The ecological advantage. *Sheep Res. J., Special Issue*: 52-64.

Weisenburger, D D (1993). Human health effects of agrichemical use. *Hum. Pathol., 24(6)*: 571-576.

Wibawa, W; Mohamad, R; Omar, D and Juraimi, A S (2007). Less hazardous alternative herbicides to control weeds in immature oil palm. *Weed Biol. Manag., 7(4)*: 242-247.

Wibawa, W; Mohamad, R; Juraimi, A S; Omar, D; Mohayidin, M G and Begum, M (2009). Weed control efficacy and short term weed dynamic impact of three non-selective herbicides in immature oil palm plantation. *Int. J. Agric. Biol., 11(2)*: 145-150.

Wibawa, W; Mohayidin, M G; Mohamad, R B; Juraimi, A S and Omar, D (2010). Efficacy and cost-effectiveness of three broad-spectrum herbicides to control weeds in immature oil palm plantation. *Pertanika J. Trop. Agri. Sci., 33(2)*: 233-241.

Zaller, J G; Weber, M; Maderthaner, M; Gruber, E; Takács, E; Mörtl, M; Klátyik, S; Győri, J; Römbke, J; Leisch, F; Spangl, B and Székács, A (2021). Effects of glyphosate-based herbicides and their active ingredients on earthworms, water infiltration and glyphosate leaching are influenced by soil properties. *Environ. Sci. Eur., 33(1)*: 1-16.

Zhao, X H; Zhang, T; Xu, M and Yao, J H (2014). Effects of physically effective fibre on chewing activity, ruminal fermentation, and digestibility in goats. *J. Anim. Sci., 89*: 501-509.

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PARAMETRIC STUDY ON TRASH SEPARATION EFFICIENCY IN FRESH FRUIT BUNCHES (FFB) CONSIGNMENT

CHE RAHMAT CHE MAT[1,2,*]; ROHAYA MOHAMED HALIM[1]; NADIAHNOR MD YUSOP[2,3]; NU'MAN ABDUL HADI[1] and ANDREW YAP KIAN CHUNG[1]

ABSTRACT

Trash or contamination in oil palm fresh fruit bunches (FFB) is one of the major problems encountered by palm oil millers. Trash is commonly referred to as non-oil palm fruit matters, such as stones, sand, soil, mud, wood, grass, leaves and twigs, which are constituted in FFB consignments delivered to the mills. Trash is undesirable as it often causes choking, severe process machinery wear and tear and reduction in oil extraction rate (OER) due to the inclusion of trash weight. In this study, a vibrating trash separating machine prototype was developed locally to separate undesirable trash from FFB consignments. This study aimed to evaluate the performance of this prototype machine based on the total amount of trash removed from the FFB and loose fruits consignments. Three gap size configurations were studied, namely 45, 30 and 15 mm. The results showed that the prototype machine successfully removed trash from wet and dry FFB consignments with separation efficiencies of 80.95% and 91.91%, respectively. The best gap size between the rod bars was indicated by 15 mm. This prototype machine is expected to solve the contamination issue during palm oil milling process due to lack of efficient system to remove trash from the FFB consignments.

Keywords: contamination, efficiency, palm oil, separation, vibrating screen.

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INTRODUCTION

Malaysia is the second largest crude palm oil (CPO) producer in the world after Indonesia, accounting for about 40% of global oil production (Otieno *et al*., 2016; Parveez *et al.,* 2020). Oil palm cultivation areas in Malaysia have grown at a rate of 3%-5% annually (MPOB, 2012). The number of palm oil mills in Malaysia has increased significantly from 352 mills in 2001 to 451 mills in 2017 (MPOB, 2017). Palm oil mills located in East Malaysia increased from 30% in 2001 to 45% in 2017 due to oil palm cultivation area expansion. Malaysia also produces 18 million tonnes of CPO annually which is equivalent to 5.64 million hectares of cultivated area (Khaled *et al.,* 2018). Palm oil mill processes oil palm fresh fruit bunches (FFB) to produce two main products, namely CPO and palm kernel (PK). It is important for Malaysian palm oil mills to extract CPO and PK efficiently for commercial purposes.

In palm oil mill, it is of utmost concern that the presence of trash or contaminants in FFB consignments is one of the major problems encountered by palm oil millers. It has been reported that the loose fruits collected by in-field harvesters through handpicking and raking contain more than 30% trash (Ahmad *et al.,* 1995; Amirshah and Hoong, 2003; Darius and Fairulnizam, 2014). In fact, Shawaludin *et al.* (1996) emphasised that the trash content in sterilised fruits, also known as mass-passing-through-digester (MPD) has increased significantly from 6% in 1990-1991 to 11% in 1995-1996. This was contributed by the calyx leaves which

[1] Malaysian Palm Oil Board,
6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.

[2] School of Chemical Engineering,
College of Engineering, Universiti Teknologi MARA (UiTM),
40450 Shah Alam, Selangor, Malaysia.

[3] Energy, Oil and Gas Unit,
Smart Manufacturing Research Institute (SMRI),
Universiti Teknologi MARA (UiTM),
40450 Shah Alam, Selangor, Malaysia.

* *Corresponding author e-mail: cherahmat@mpob.gov.my*

comprised up to 33% of trash. The remaining 67% of trash consisted of sand, stones, mud and other plant materials. The trash including non-oil palm fruit matters, such as stones, sand, soil, mud, wood, grass, leaves and twigs, are often ignored by millers and other stakeholders (The Edge Markets, 2018).

Trash accumulated in FFB consignments causes problems during milling process. For example, the presence of excessive abrasive sand particles in the FFB will contribute to frequent process flow choking and excessive process machinery wear and tear, thereby increasing the operating and maintenance costs of critical machineries, such as digester, screw press, conveyor and centrifuge. In addition, stones and other objects in FFB can disrupt processing, which in turn reduces mill throughput effectively. Most importantly, trash also tends to absorb some part of the mesocarp oil, thus, reducing the efficiency of the oil extraction process (Badmus *et al.*, 2005). Consequently, it lowers the oil extraction rate (OER) of palm oil mill.

Recently, findings on the adverse effect of 3-monochloropropane-1, 2-diol (3-MCPD) esters as contaminants in refined palm oil have jeopardised the palm oil industry. It has been reported that the deodourising temperatures in refining process, pH level and chloride content in CPO production are the contributing factors to the 3-MCPD esters formation in refined palm oil (Ibrahim *et al.*, 2012; 2016). Nonetheless, there is no conclusive evidence to suggest that the source of chloride is actually originated from CPO. On the contrary, it may stem from the FFB consignment containing sand, soil and mud from the field. During harvesting, oil palm fruits that fall to the ground and FFB harvested tend to mix with the trash (sands, muds, leaves, fertiliser). Trash embedded in FFB is usually processed together during sterilisation process (*Figure 1*) as FFB cleaning before processing is not currently practiced at the mill. The use of chloride-containing fertiliser could also cause chloride contamination in FFB.

Figure 1. Trash mixed with oil palm bunches and loose fruits in a cage before sterilisation process.

Furthermore, there is limited equipment or system to remove trash efficiently during milling process such as sand catchers, de-stoners and de-sanders. Additional manpower is normally needed to address problems caused by extraneous objects in FFB. Although loose fruit collecting machine can reduce the trash amount, the acceptance of the use of this machine by planters is still low due to technical limitation and expensive cost (Khalid and Shuib, 2017). Ropandi and Zulkifli (2002) reported that the use of MPD washing system was able to remove sand and stone about 71.4% on average. With exclusion of stones, the removal percentage apparently reached as high as 93.0%, indicating an excellent and achievable target.

Moreover, FFB hopper was previously designed with slots on an inclined surface and equipped with a rail section to enable trash to pass through the gap. However, small fruitlets were discarded together with the trash, which required adjustment in the size of the openings to achieve effective separation. Nevertheless, high construction and maintenance cost, frequent hopper gap clogging and high loss of small loose fruits resulted in poor and outdated design. In current milling practice, the segregation and removal of trash are carried out by slotting one of the FFB conveyors as shown in *Figure 2*. FFB and trash pass through without an efficient mechanical separation system. As a result, the quantity of trash removal is about 60-80 kg per day or less. Meanwhile, *Figure 3* shows the previous design of oil palm fruit screening developed by Badmus *et al.* (2005).

Currently, palm oil mills are designed to remove trash progressively during processing via crude oil de-sander, dilution tank, vertical clarifier tank, continuous settling tank, nut polishing drum, de-stoner, sludge separator and decanter. With the presence of trash, the machinery damage in the processing line is inevitable. Thus, it is critically needed to remove trash at the initial processing stages to prevent excessive wear and tear to the processing machinery by addressing the root cause of the problem. To date, the segregation of trash from FFB consignment has not been well practiced in palm oil mills. It is necessary to improve separation efficiency by installing an efficient mechanical separation system. Thus, this study is aimed to highlight the development of a vibrating trash separator machine (TSM) as a prototype to remove trash from the FFB consignment prior to milling process.

In this study, the performance of the prototype machine was evaluated based on total amount of trash removed from the loose fruits and FFB consignments through the difference of gap sizes between the rod bars. The screening design was made based on the trash size so that appropriate openings for trash aperture can be obtained. Trash can be divided into various sizes based on minimum loose fruit size to force the particles through a

specific screen size (Enrique *et al*., 2005). The best gap size between the rod bars was determined based on trash removal efficiency of dry and wet FFB consignments, and loss of loose fruits. This study is expected to promote good milling practices in maintaining uncontaminated FFB consignment and provide better trash management in palm oil mills.

Figure 2. Separation unit at FFB conveyor for trash removal.

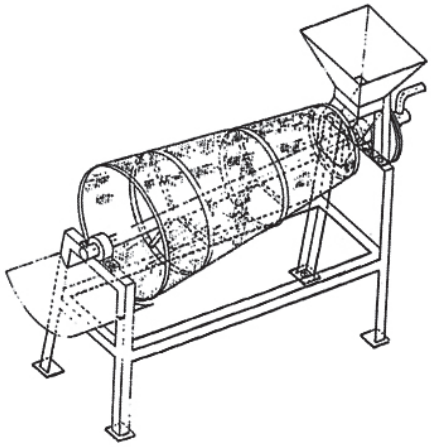


Figure 3. Oil palm fruit screening machine.

MATERIALS AND METHODS

Trash Separator Machine (TSM) Prototype

A prototype for an industrial vibrating TSM was developed to meet the actual palm oil mill operation, which included a pre-cleaning system to remove trash from FFB consignments. This TSM was constructed and installed without altering the current milling operation. It was used to reduce the weight of FFB processed by removing trash from the initial processing stage as pre-cleaning system. The designed TSM components consisted of sieve, shafts, pulley (for shaft and electric motor), bearing, belt, spring, electric motor and support.

The TSM was also designed to separate non-oil palm fruit matters from FFB consignments such as sands, soil, mud, stones and leaves. *Figure 4* illustrates the schematic diagram of TSM with 2100 mm width and 4572 mm length. *Figure 5* illustrates the prototype of the vibrating TSM. The bars were made of mild steel with various cross-sectional profiles. Round and hollow cross-section rod bars were installed as each bar was mounted individually onto the transverse beam. The vibrating sieve was equipped with 25 mm diameter springs on the side of the vibrating sieve unit. The unit was also configured with high frequency and 30 kW electric motor unit.

Materials

FFB samples consisting of loose fruits and trash were obtained from a nearby plantation in Teluk Intan, Perak, Malaysia. Most of the collected samples were of the *Tenera* species; a crossbreed clone between *Dura* and *Pisifera* which has 30% more mesocarp for high oil production (Babu *et al*., 2017). FFB and loose fruits received were from three maturity phases, namely unripe, underripe and ripe. The ripeness standard was determined based on the grading method developed by Malaysian Palm Oil Board (MPOB, 2016).

To represent dry consignment, the FFB and loose fruit samples were collected randomly on sunny days (not raining the previous day), while the wet consignment was represented by samples collected on rainy days. The FFB consignments comprised of oil palm bunches, loose fruits and trash, weighing in the range of 1000-1500 kg. These consignments were used in this study to evaluate the efficiency of the developed TSM for trash removal.

In addition, a total of 100 kg contaminated loose fruits samples was also collected from the plantation using rake and hand-picked. These samples were used to determine the best gap size to retain loose fruits from being removed with trash. Contaminated loose fruit samples were sorted manually into only trash and only loose fruits, and then weighed. Based on the weight of the sorted trash and loose fruits, the best gap size was determined.

Performance Evaluation of TSM

The performance of the TSM was evaluated in terms of trash separation efficiency and capacity by considering two parameters. The trash separation efficiency of this study was based on actual on-going and periodic measurements. The first parameter studied was the variation of the gap size between the bar rods of the TSM to allow trash to pass through the system, and the second parameter was to obtain the best gap size for high separation efficiency of dry and wet FFB consignments.

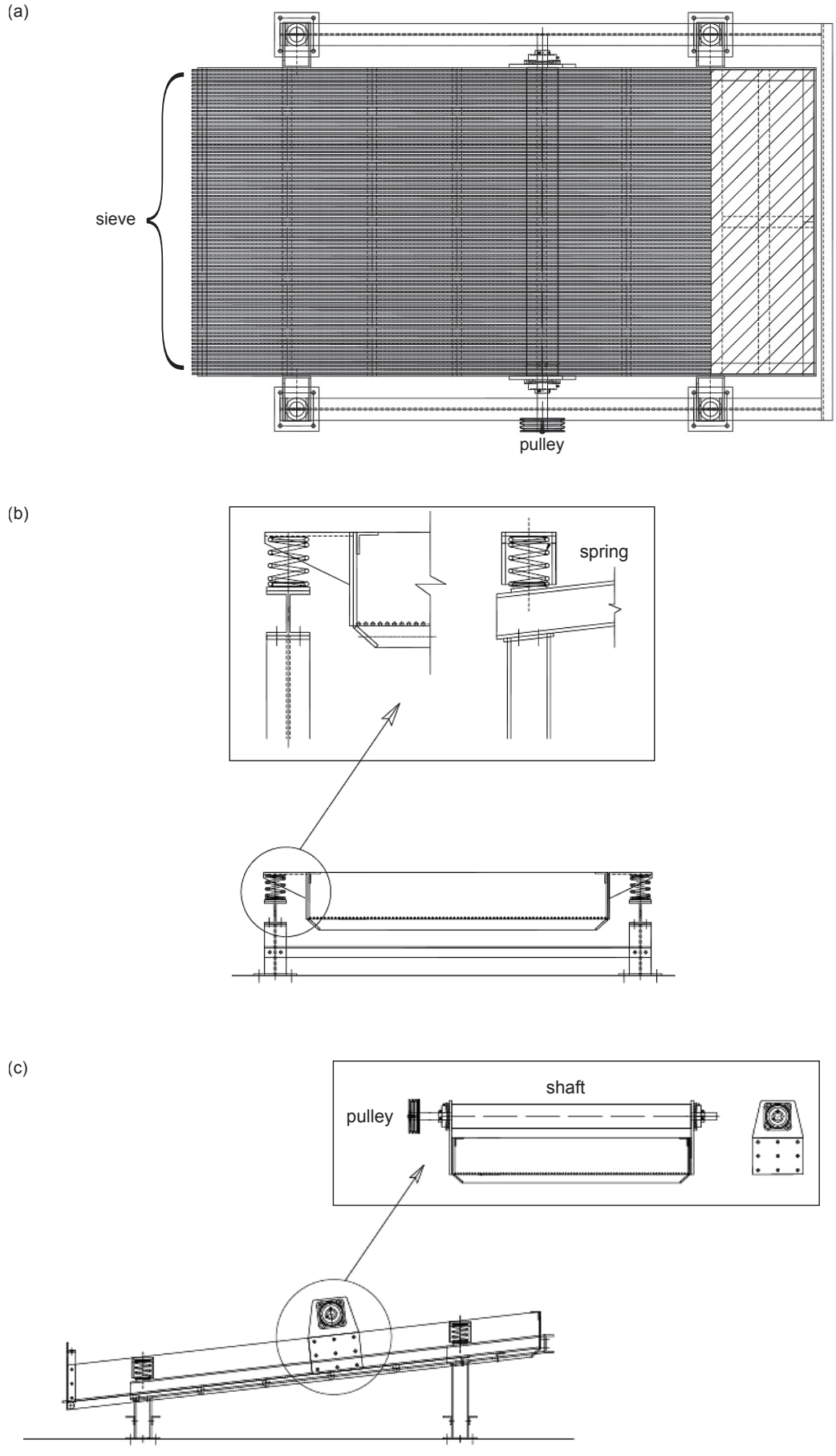


Figure 4. Schematic diagram of vibrating TSM (a) top view, (b) front view and (c) side view.

Figure 5. Prototype of vibrating TSM.

Determination on Effect of Different Gap Size

The TSM feed was fitted with various sizes of loose fruits and trash. About 100 kg of contaminated loose fruits consignment passed through the TSM to remove sand, stone, vegetative matters and other trash. The TSM was also tested for its efficiency to remove maximum amount of trash. Three different gap sizes of 45 mm, 30 mm and 15 mm were investigated for trash separation from the loose fruit consignments. The largest gap size of 45 mm was selected which corresponded to the thresher drum slot size of 46 mm (Leang, 2011). The thresher drum serves to remove sterilised oil palm fruitlets from the bunches during the milling process.

Selection of the gap size was based on the average diameter and length of oil palm fruitlets. The average diameter and length of oil palm fruitlets are between 18 to 25 mm and 35 to 40 mm, respectively (Basyuni *et al*., 2017). The separation gap size between the rod bars was correlated to the smallest loose fruit size to minimise loss of fruitlets during the separation process. The gap size was reduced from 45 mm to a minimum size of 15 mm to investigate the efficiency of the separation process. The smallest gap size selected was 15 mm due to manufacturing constraint and availability. Six repetitions were performed to variations in gap size for loose fruit consignments.

Determination on Trash Separation Efficiency of Dry and Wet FFB Consignment

Figure 6 illustrates the separation process flow of the TSM to remove contaminants from the FFB consignments. The consignments consisting of loose fruits and trash as received from nearby plantation were weighed and unloaded from the lorry onto the loading ramp at the mill. Then, the dry or wet FFB consignment was subjected to separation process by passing through the TSM, which served as a pre-cleaning system to remove sand, stone and old loose fruits prior to processing to ensure maximum trash removal.

The TSM was fixed at an inclination of 15° to allow smooth flow of FFB through the sieve under gravitational effect. It was found that at lower inclination degree (less than 15°), the FFB flow was halted causing backlog of bunches at the entry to the TSM. At inclination degrees higher than 15°, FFB was separated smoothly but the removal of trash was not efficient. This could be due to short retention time for the FFB to pass through the TSM.

In this study, it was assumed that the loss of loose fruits together with trash did not occur to facilitate the measurement of the separation efficiency of trash from the FFB consignment. Separation efficiency, (S_e) is defined as the ratio of the amount of trash separated by the TSM screening to the amount of trash to be separated loaded into the TSM screening. Constant trash weight was added manually to the FFB consignment before being fed into the TSM. The trash removed from the TSM was collected, analysed and quantified. The calculation of S_e of dry and wet consignments is shown in Equation (1).

$$S_e = \frac{Weight\ of\ trash\ separated}{Weight\ of\ trash\ added} \times 100 \qquad \text{Equation (1)}$$

Statistical Analysis

Data for mean, separation efficiency, standard deviation, standard error and confidence intervals were analysed statistically using Microsoft Excel. The paired *t*-test as expressed in Equation (2) was used to test for significant difference between the

amounts of trash separated in the two consignment conditions (*i.e.*, dry and wet) studied.

$$t = \frac{d}{s\sqrt{1/n}} \qquad \text{Equation (2)}$$

where,
d = average difference in weights of trash separated between two conditions
s = standard deviation
n = sample population

The mean weight of trash was calculated by Equation (3):

$$x = \frac{\Sigma xi}{N} \qquad \text{Equation (3)}$$

where,
Σxi = sum of trash weight
N = number of trial sample

RESULTS AND DISCUSSION

Trash Contamination in FFB Consignment

Trash contamination in FFB consignment is categorised as trash embedded in FFB and trash mixed with loose fruits (*Figure 7a*). Trash embedded in FFB could be due to the impact when fruit bunches fall to the ground during harvesting. *Figure 7b* illustrates trash mixed with loose fruits during on-site collection and transportation.

Effect of Different Gap Sizes

The effect of gap sizes on trash separation efficiency with loose fruit consignment is summarised in *Table 1*. During the observation, trash was sent to the palm oil mill together with loose fruits consignment containing high amount of sand, soil, mud, stones and vegetative matter. In fact, most of them were in the form of calyx leaves as shown in *Figure 8*. *Table 1* indicates the performance of the

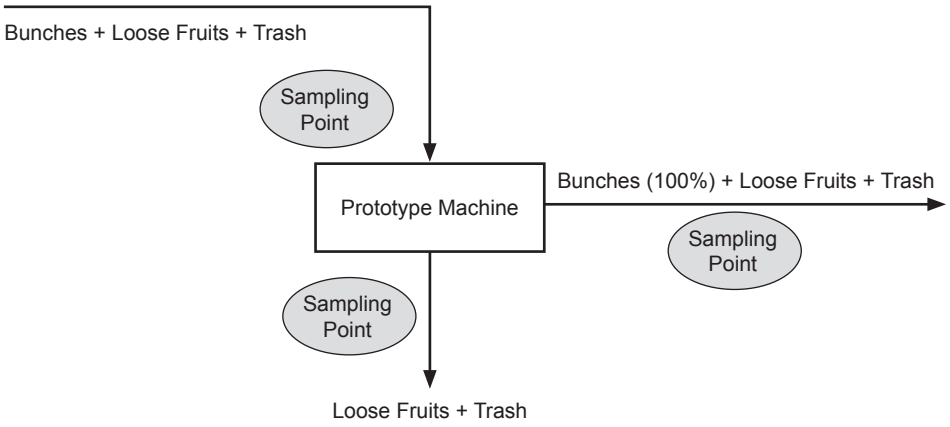


Figure 6. Process flow to separate trash from FFB consignment.

Figure 7. Trash mixed with (a) FFB and (b) loose fruits.

separation process for the TSM. It can be observed that as the gap size decreased, the separation efficiency improved. The amount of trash passing through the screen as well as the amount of loose fruits carried over into the trash consignment with different gap sizes of rod bars were recorded.

Figure 8. Non-oil palm fruit matter collected after screening.

More trash was removed at a gap size of 45 mm between the rod bars. The average amount of trash that can be removed was about 35.03 ± 1.09 kg and suffered high loss of loose fruits carried over with trash. The amounts of trash removed for 30 mm and 15 mm gap sizes were about 17.86 ± 1.66 kg and 6.46 ± 1.29 kg, respectively. The amount of trash weighed included the weight of loose fruits removed together with trash during screening. Each gap size was tested six times with different loose fruit consignments to ensure different loose fruits and trash sizes passed through the TSM during screening.

The amount of trash removed decreased as the gap size between the rod bars decreased from 45 to 15 mm. It was also observed that the separation efficiency for removing trash from loose fruits increased when less loose fruits were carried over during screening. This may be influenced by the decreasing gap size between the rod bars.

Due to various trash sizes of present in loose fruit consignments, TSM screening was developed with small gap sizes to allow small stones, grass, twigs and sand to pass through. This is in agreement with Davies (2012) who reported that the geometric and arithmetic mean diameters of loose fruits ranged from 21.36 to 29.23 mm and 20.80 to 27.80 mm, respectively.

However, such design also allowed some loose fruits to pass through which resulted in high losses to palm oil mill. Therefore, the recovery of loose fruits is prioritised to remove trash optimally at this stage. Loose fruit recovery is a critical factor as loose fruits come from the outer layer of FFB and account for up to 50% of total percentage of oil to bunch (Gan *et al.*, 1995).

Trash Separation Efficiency on Dry and Wet FFB Consignment

Separation efficiency for removal of non-oil palm fruit matter in dry and wet FFB consignments is shown in *Table 2*. A paired *t*-test was performed to analyse the resulting trash weights separated for both consignment conditions. The results indicated that there was no significant difference between the two sets of FFB consignments (wet and dry). There was a tendency towards a decreasing trend of separation efficiency for dry and wet FFB consignments.

TABLE 1. EFFECT OF GAP SIZES ON TRASH SEPARATION PERFORMANCE

	Gap size		
	45 mm	**30 mm**	**15 mm**
Weight of sample (kg)	100	100	100
Mean weight of trash removed (kg)	35.03	17.86	6.46
Standard deviation, S_d	1.09	1.66	1.29
Observations	Contained many loose fruits in various sizes	Contained some loose fruits in various sizes	Contained few loose fruits

TABLE 2. SUMMARY OF TRASH SEPARATION EFFICIENCY OF DRY AND WET FFB CONSIGNMENT

	Dry FFB consignment			Wet FFB consignment		
Weight of FFB consignment (kg)	500	550	600	500	550	600
Weight of trash added (kg)	40	50	60	60	50	40
Weight of trash removed (kg)	36.51	45.88	55.63	48.81	40.29	32.37
Trash separation efficiency (%)	91.28	91.76	92.72	81.35	80.58	80.93

For dry consignment, the separation efficiency ranged from 91.28% to 92.72%, while the separation efficiency for wet consignment ranged from 80.58% to 81.35%. The highest separation efficiency (92.72%) was obtained at 600 kg dry consignment, while the lowest efficiency (80.58%) was obtained at 550 kg for slightly wet consignment. Separation efficiency decreased by 12.14% depending on the consignment conditions.

The wet consignment was affected by rain and this condition made the trash more sticky and easily embedded in the FFB. Rain water can also increase the moisture content of FFB and reduce the quality of CPO (Ayat *et al*., 2009). This complicated the process of removing trash from the FFB consignment, especially after rainy days. The vibration mechanism integrated in the TSM forced the mud to detach from the FFB which resulted in high separation efficiency achievable for both conditions (wet and dry). Thus, it can be inferred that the TSM was able to separate trash with efficiency of more than 80%.

Referring to *Table 2*, the results also revealed that the TSM was able to achieve an average separation efficiency of 91.91 ± 0.73% for trash removal for dry consignment. The average trash removal separation efficiency for wet consignment decreased slightly to 80.95 ± 0.39%. A similar study by Badmus *et al.* (2005) showed that calyx can be removed from oil palm fruits after threshing with separation efficiencies of 82% and 96% for wet and dry consignments, respectively.

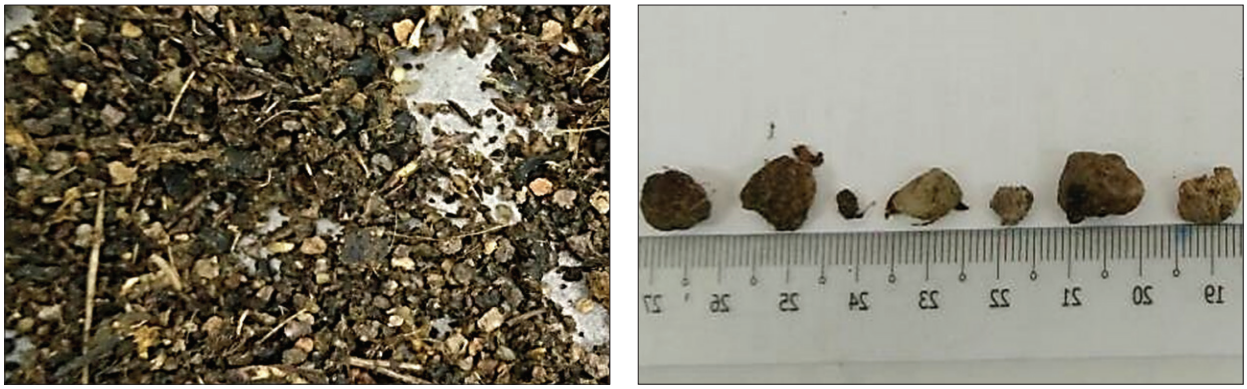
Results showed that the FFB consignment conditions (dry or wet) had an influence on the separation efficiency. Wet trash, such as sand and soil embedded in the bunch in the form of mud, absorbed more water and sticked to the FFB. Such trash was difficult to separate or remove. This is in agreement with Folami *et al.* (2016) who also found that the separation efficiency was low for rice processing machine as the feeding material has high moisture content. Similar results were also reported by Firouzi *et al.* (2010) where the performance of rubber roll husker decreased as the moisture content of paddy increased.

Performance Evaluation of TSM

Trash removal efficiency of 1000 kg FFB consignment is summarised in *Table 3*. The results obtained in this study were based on the actual process that was on-going with the gap size of 15 mm and fixed inclination of 15° installed at the palm oil mill. The trials were repeated with 15 samples of FFB consignment samples for each condition. From *Table 3*, the relationship between separation efficiency, and wet and dry consignments was observed. The collected of trash after TSM as shown in *Figure 9.* The results showed that wet consignment has lower separation efficiency compared to dry consignment. This TSM was able to remove trash from dry consignment as much as 7.63 ± 0.70 kg from 1000 kg dry consignment. The results of this study also showed that 95% confidence intervals for trash removal ranged from 6.26 kg to 8.99 kg. Meanwhile, trash removed from wet consignment was 5.50 ± 0.45 kg per 1000 kg FFB with trash removed from wet consignment recorded 95% confidence interval ranging from 4.62 kg to 6.38 kg.

Stone removal is important for the mill to reduce machine wear and tear during processing. *Table 4* summarises the amounts of small stones that were removed during the trial. This indicated that about 0.37-0.53 kg of small stones were removed using the TSM for each tonne of FFB (*Figure 10*) which was approximately equivalent to 5.86%-7.49% of the total trash removed. The remaining 92.51%-94.14% of trash consisted of sand, mud and vegetative matter. Normally, mill processes FFB around 500-1000 t of FFB per day based on the mill capacity. Hence, the result shown that for 1000 t of FFB consignment processed was contained about 435.84 kg of small stones that can affect the machine wear and tear during processing.

Figure 9. Foreign material and trash collected after separation process of TSM.



(a) (b)

Figure 10. (a) Stones and (b) sizes of stones collected after separation by TSM.

TABLE 3. ANALYSIS FOR 1000 KG FFB FED TO TRASH SEPARATION MACHINE

Type of feed	Weight (kg)				
	FFB	Mean	S_d	S_{err}	95% CI
Dry	1 000	7.63	0.70	0.18	6.26 - 8.99
Slightly wet	1 000	5.50	0.45	0.12	4.62 - 6.38

Note: S_d - standard deviation; S_{err} - standard error; CI - confidence intervals.

TABLE 4. QUANTITY OF STONES IN FFB DELIVERED TO PALM OIL MILL

Trial	FFB passed through (t)	Small stone removed (kg)	FFB (kg t^{-1})
1	200	75.08	0.370
2	200	106.26	0.530
3	200	84.94	0.420
4	200	90.82	0.450
5	200	78.74	0.390
Total	1 000	435.84	-

During separation of trash, mud from wet consignment tended to accumulate on the TSM rods. This affected the separation efficiency owing to the reduction of space between the rod bars to remove the trash. The efficiency of physical separation depends on various parameters such as moisture content, clay content, humid content, particle shape and size distribution (Balasubramaniam, 2017; USEPA, 1995; Williford *et al.*, 2000). These parameters should be taken into account when attempting to predict screening performance.

This study also found that the fruitlets were prone to bruising during screening as the FFB collided with the conveyor prior to screening. Previous studies have postulated that free fatty acid (FFA) may increase with bruising levels rather than time delay between bruising and oil production (Che Rahmat *et al.*, 2018; Hadi *et al.*, 2009). In addition, Rasli *et al.* (2019) deduced that crop parameters and loading orientation affect the rupture force and energy of oil palm fruitlets which decrease with ripeness.

It is expected that this innovation will boost the efficiency of CPO production and increase the OER of palm oil mill by reducing the weight of FFB processed. It can also solve the oil production and quality problems by removing trash and preventing wear and tear of machinery at the initial stage of the milling process.

CONCLUSION

This study demonstrated the capability of vibrating TSM to remove significant amount of trash prior to milling process. The results of this study concluded that the efficiency of the TSM to remove trash from

the wet FFB consignment was lower than that of dry consignment. The gap size of 15 mm was found to be able to remove trash from dry and slightly wet FFB consignments with average separation efficiencies of 91.91% and 80.95%, respectively, with minimum loss of loose fruits. This TSM can benefit palm oil millers in terms of weight reduction between 5.50-7.63 kg per 1000 kg FFB consignment processed. The deduction of contaminant weight from total amount of FFB processed will consequently increase the OER. Better oil recovery is also expected as oil losses normally absorbed by foreign vegetative materials have been minimised. The vibrating TSM performance showed that the screening can be utilised by palm oil millers as a pre-cleaning system for their initial processing stage. Moreover, the machinery wear and tear can be reduced by preventing trash from entering the processing line.

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REFERENCES

Ahmad, H; Ahmad Zamri, M Y and Mohd Salih, J (1995). Loose fruit collector. *PORIM Information Series No. 19*. http://palmolis.mpob.gov.my/publication/TOT/TT-19.pdf.

Amirshah, T and Hoong, H W (2003). Improved loose fruit collection system using sawit loose fruit separator. *Proc. of the PIPOC 2003 International Palm Oil Congress – Agriculture Conference.* MPOB, Bangi. p. 43.

Ayat, K A R; Ramli, A; Mohd Arif, S and Faizah, M S (2009). Management of the Malaysian oil palm supply chain: The role of FFB dealers. *Oil Palm Industry Economic J., 9(1)*: 20-28.

Babu, B K; Mathur, R K; Kumar, P N; Ramajayam, D; Ravichandran, G; Venu, M V B and Sparjan Babu, S (2017). Development, identification and validation of CAPS marker for SHELL trait which governs *dura, pisifera* and *tenera* fruit forms in oil palm (*Elaeis guineensis* Jacq.). *PLoS ONE, 12(2)*: e0171933.

Badmus, G A; Adeyemi, N A and Owolarafe, O K (2005). Performance evaluation of an oil palm fruit screen. *J. Food Eng., 69*: 173-176.

Balasubramaniam, A (2017). Ore separation by classification. https://doi.org/10.13140/RG.2.2.32055.52644, accessed on 24 December 2020.

Basyuni, M; Amri, N; Putri, L A P; Syahputra, I and Arifiyanto, D (2017). Characteristics of fresh fruit bunch yield and the physicochemical qualities of palm oil during storage in North Sumatra, Indonesia. *Indones. J. Chem., 17(2)*: 182-190.

Che Rahmat, C M; Nu'man, A H; Rohaya, M H and Rusnani, A M (2018). Evaluation of double-roll crusher on oil palm fruit bunches and effect of bruising level on free fatty acid content. *J. Eng. Sci. Technol., 13(10)*: 3381-3392.

Darius, E P and Muhammad Fairulnizam, H (2014). Effects of collecting systems and plantation environment on debris accumulation in a collected oil palm loose fruits. *Proc. Int. Conf. Plant. Physiol.*, 2014. Bali, Indonesia. p. 147-151.

Davies, R (2012). Physical and mechanical properties of palm fruit, kernel and nut. *Int. J. Agric. Technol., 8(7)*: 2147-2156.

Enrique, O R; Juliano, P and Yan, H (2005). Separation and classification. *Food Powders: Physical Properties, Processing and Functionality*. Kluwer Academic/Plenum Publishers, New York, USA. p. 247-255.

Folami, A A; Obioha, E N; Adewole, A A and Ibiyemi, K S (2016). Performance evaluation of a developed rice-processing machine. *J. Agric. Eng., 47(3):* 171-176.

Firouzi, S; Alizadeh, M and Minaei, S (2010). Effect of rollers differential speed and paddy moisture content on performance of rubber roll husker. *Int. J. Nat. Eng. Sci., 4(3)*: 37-42.

Gan, L T; Ho, C Y; Lam, K S and Chew, J S (1995). Optimum harvesting standards to maximize labour productivity and oil recovery. *Proc. of the 1993 PORIM Int. Oil Palm Congress - Update and Vision*. Kuala Lumpur. p. 195-211.

Hadi, S; Ahmad, D and Akande, F B (2009). Determine of the bruise indexes of oil palm fruits. *J. Food Eng., 95*: 322-326.

Ibrahim, N A; Ramli, M R; Abdul Razak, R A and Kuntom, A (2016). 3-MCPD Esters: A new challenge for the palm oil industry. *Proc. of the 2016 National Seminar on Palm Oil Milling, Refining, Environment and Quality (POMREQ).* MPOB, Kuala Lumpur.

Ibrahim, N A; Ramli, M R and Lin, S W (2012). Possible factors that cause the formation of 3-monochloropropane-1, 2-diol (3-MCPD) esters. *Palm Oil Developments*, *57*: 1-6.

Khalid, M R M and Shuib, A R (2017). Performance of oil palm loose fruits separating machine. *J. Oil Palm Res.*, *29(3)*: 358-365.

Khaled, A Y; Aziz, S A; Bejo, S K; Nawi, M N; Seman, I A and Izzuddin, M A (2018). Development of classification models for basal stem rot (BSR) disease in oil palm using dielectric spectroscopy. *Ind. Crops Prod.*, *124*: 99-107.

Leang, L H (2011). Removal of abrasive materials from FFB before milling. *Palm Oil Eng. Bull., (103)*: 21-31.

MPOB (2012). Malaysian palm oil industry. http://palmoilworld.org/about_malaysian-industry.html, accessed on 1 February 2020.

MPOB (2016). *Manual Penggredan Buah Kelapa Sawit*. Third edition. MPOB, Bangi. 42 pp.

MPOB (2017). *Directory of Malaysian Palm Oil Processing Sectors*. 6th edition. MPOB, Bangi. 136 pp.

Otieno, N E; Dai, X; Barba, D; Bahman, A; Smedbol, E; Rajeb, M and Jaton, L (2016). Palm oil production in Malaysia: An analytical systems model for balancing economic prosperity, forest conservation and social welfare. *Agric. Sci., 7(2)*: 55-69.

Parveez, G K A; Elina, H; Soh, K H; Meilina, O A; Kamalrudin, M S; Mohd, N I Z Z B; Shamala, S; Zafarizal, A A H and Zainab, I (2020). Oil palm economic performance in Malaysia and R&D progress in 2019. *J. Oil Palm Res., 32(2)*: 159-190.

Rasli, A M M; Nawi, N M; Ahmad, D and Yahya, A (2019). The effect of crop parameters on mechanical properties of oil palm fruitlets. *Sci. Hortic., 250*: 352-358.

Ropandi, M and Zulkifli, A (2002). Removal of trash in sterilized fruitlets in palm oil mill. *MPOB Information Series No. 164*.

Shawaludin, T; Japri, G and Hashim (1996). A practical approach to improving OER - Golden Hope experience. *National Seminar on Opportunities for Maximizing Production through Better OER and Offshore Investment in Oil Palm*. PORIM, Bangi. p. 268-271.

The Edge Markets (2018). The problems with palm oil. https://www.theedgemarkets.com/article/problems-palm-oil, accessed on 14 January 2020.

USEPA (1995). Contaminants and Remedial Options at Selected Metal-Contaminated Sites. EPA/540/R-95/512. United States Environmental Protection Agency. Washington DC.

Williford, C W; Bricka, R M and Iskandar, I (2000). Physical separation of metal-contaminated soils. *Environmental Restoration of Metals-Contaminated Soils*. CRC Press LLC, Boca Raton. p. 121-165.

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MICROBIAL PROTEIN EXTRACTION FROM PALM OIL MILL EFFLUENT

NOR FAIZAH, J[1]*; NOORSHAMSIANA, A W[1]; WAN HASAMUDIN, W H[1]; NAHRUL HAYAWIN, Z[1]; ROPANDI, M[1]; NUR ELIYANTI, A O[1]; ASTIMAR, A A[1] and ROHAYA, M H[1]

ABSTRACT

*Palm oil mill effluent (POME) is abundantly produced during palm oil milling process and is treated for pollutant reduction without any revenue or profit. The main objective of this research was to evaluate POME as a substrate for microbial protein production and analyse the removal rate of pollutant. This study used hydrolysed POME containing fermentable sugars as carbon source for the cultivation of microbes. Raw POME was initially pre-treated with Celluclast® enzyme using the following conditions: Concentration (1.5% to 5.0% v/v); incubation temperature (45°C-55°C), rotation speed (100-200 rpm) and pH (4.0-5.5). After pre-treatment, the hydrolysate contained 41.63 g L^{-1} reducing sugars with 56 000 mg L^{-1} chemical oxygen demand (COD). Baker's Yeast (*Saccharomyces cerevisiae*) was then cultivated onto the raw or hydrolysed POME at 30°C for seven days. The amount of yeast biomass produced was 28.92 g L^{-1} with 24.79% protein content. Adding to this, the COD value was reduced by 79.12%. The yeast fermentation in hydrolysed POME recorded the highest increase in biomass and protein contents of 3.44 and 7.74 folds, respectively. The findings revealed that POME is a promising raw material for microbial biomass protein production and simultaneously remove the pollutant from POME.*

Keywords: biomass, microbial, palm oil mill effluent, single cell protein.

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INTRODUCTION

Malaysia processed more than 97.8 million tonnes of fresh fruit bunches (FFB) in 2018 (Kushairi *et al.*, 2019). Apart from the production of crude palm oil, the mills also generated enormous amount of palm oil mill effluent (POME) every year. In general, 1 t FFB would generate 0.67 m^3 POME (Ma, 1999). As a result, more than 65 million m^3 POME was discharged from the mills. Without treatment, raw POME is a polluting material with high chemical oxygen demand (COD) (51 000 mg L^{-1}), biological oxygen demand (BOD) (25 000 mg L^{-1}) and total suspended solids (TSS) (18 000 mg L^{-1}) (Nahrul Hayawin *et al.*, 2017).

Raw POME is basically a dark brown oily waste which consists of carbohydrate, protein, nitrogen (N), lipids, minerals and other nutrients. Dried POME contains 39.3% cellulose and 24.6% hemicellulose (Khaw *et al.*, 2008). Conventionally, POME is treated using the open pond system consisting of a cooling pond, an acidification pond, and the anaerobic and aerobic ponds. However, this system has its limitation where the BOD value is merely managed to reduce from 25 000 mg L^{-1} to 50-100 mg L^{-1}. Many advanced technologies on POME treatment are being introduced including membrane ultrafiltration (Numan *et al.*, 2019), membrane nanofiltration (Ali Amat *et al.*, 2015), electrocoagulation (Nasrullah *et al.*, 2018), and a hybrid system that combines the activated carbon adsorption and ultrasound cavitation (Parthasarathy *et al.*, 2016). However, these advanced systems require very high capital and operating costs without returning any profits to the palm oil millers although they are very effective in reducing BOD, COD, TSS and colour.

[1] Malaysian Palm Oil Board,
6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.

* *Corresponding author e-mail: norfaizah@mpob.gov.my*

Alternatively, these waste streams can be converted from an economic liability into a source of revenue. POME could be converted via bioconversion into value-added products such as bioenergy and biochemical. The high compositions and concentrations of carbohydrate, protein, N, lipids, minerals and other nutrients in POME make it possible to reuse the effluent for biotechnological processing (Nurul Adela *et al.*, 2020). Therefore, researchers are starting to propose on recovering high value products from POME in order to increase the revenue of the palm oil millers and at the same time will promote sustainable oil palm industries (Chia *et al.*, 2020). The recovery processes mostly practised in mills include biogas capture and biomass co-composting. Numerous attempts have been made to utilise POME in composting such as through vermicomposting and co-composting with empty fruit bunch (EFB) and decanter cake (Nahrul Hayawin *et al.*, 2017). Another sustainable approach on POME utilisation is as a substrate for microbial fermentation and cultivation. For instance, microalgae can be cultivated in POME for bioremediation as well as a feedstock for biodiesel production (Kamyab *et al.*, 2015). Furthermore, hydrolysed POME could be used to produce bioflocculant via POME-isolated *Bacillus marisflavi* cultivation. The resulted bioflocculant is potentially be used for wastewater treatment and algae recovery (Nurul Adela *et al.*, 2016; 2020).

Another potential approach for resource recovery from POME is for production of single cell protein (SCP). SCP refers to sources of mixed proteins extracted from pure or mixed cultures of microorganisms. It is one of high quality dietary products for animals feed such as ruminants, pig, chicken and aquaculture application that enable to replace soymeal and fishmeal due to its high protein and nutrient content (Patelski *et al.*, 2020; Sharif *et al.*, 2021). Crude SCP which is also termed as bioprotein, microbial protein or biomass are widely produced from agricultural waste sources such as molasses, dairy waste, fruit waste, starch rich waste, bran, poultry waste *etc.* (Spalvins *et al.*, 2018). SCP produced from different microbes has high protein content (30%-70%) as compared to different green plants and animal sources. Previously, several types of microorganisms have been studied for the production of SCP such as yeast, bacteria, fungi and algae (Anupama and Ravindra, 2000). Microorganisms that are widely used in SCP production include *Candida utilis* and *Saccharomyces cerevisiae* since both microorganisms are safe for consumption. For instance, *S. cerevisiae* that is produced in undiluted POME is able to produce 4.42 g L^{-1} protein biomass with 27% protein content. Another study by Izah (2018) highlights the cultivation of *S. cerevisiae* in palm oil and cassava mill effluent. Based on proximate composition the results are also promising especially with protein content which is higher than 17% but with low yield (approximately 4 g L^{-1}).

Since POME contains insoluble carbohydrates with high molecular weight compounds such as cellulose, hemicellulose and starch, it requires pre-treatments to hydrolyse the complex carbohydrates in POME prior to fermentation to aid or speed up the microbial process (Nurul Adela *et al.*, 2016). Khaw *et al.* (2008) has determined that POME solid contains about 39.3% cellulose and 24.6% of hemicellulose and these can be converted to fermentable sugars through hydrolysis process. Pre-treatments could disrupt the structure of lignocellulosic materials, hence releasing more reducing sugars which enhance the production of biochemicals. For instance, the sugar content in POME treated with cellulase enzyme increased from 12.45 to 27.13 g L^{-1} (Nurul Adela *et al.*, 2016). Xylanase enzyme enables the release of sugars from hemicellulose in POME prior to biogas production and improves the hydrolytic reaction that increases the methane production (Prasertsan *et al.*, 2017). Cellulolytic fungi such as *Trichoderma harzianum* and *Mucor hiemalis* are added in order to convert carbohydrate polymers in POME into fermentable sugar prior to yeast fermentation for bioethanol production (Alam *et al.*, 2009). It was also reported that alkaline pre-treatment by using 2.58% sodium hydroxide (NaOH) could increase the fermentable sugar content by 9.35% relative to untreated POME (Izzi *et al.*, 2020). This showed that alkaline pre-treatment is also one of viable pre-treatment methods in addition to enzymatic hydrolysis method.

However, exploration of POME as a substrate for microbial protein production is still lacking compared to bioenergy and microalgae. To date, very limited studies are available on the utilisation of POME for SCP production (Iwuagwu and Ugwuanyi, 2014). Therefore, the aim of this article was to evaluate POME, either in raw or hydrolysed form, as potential material for obtaining the yeast biomass and producing microbial protein as a crude SCP. The pollutant removal will also be quantified in terms of COD reduction. This approach may recover and utilise value-added products from the palm oil mills, particularly from POME before being discharged into water courses. Apart from generating profits, this approach will indirectly help to safeguard our environment.

MATERIALS AND METHODS

Raw Materials

Samples of mixed raw effluent (MRE) containing steriliser condensate and clarification underflow sludge were collected at the sludge pit

from Palm Oil Mill Effluent Technology Centre (POMTEC), located in Negeri Sembilan, Malaysia. These samples were transported and kept under 4°C.

Cellulase (Celluclast® 1.5 L FG) used in MRE hydrolysis was obtained from Novozyme (M) Sdn. Bhd. The enzyme is commercially produced from *Trichoderma reesei* with the activity of 700 endo-glucanase units (EGU) g^{-1}.

Dry yeast (Mauripan) was inoculated onto a basal medium consisting of glucose (50 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.025 g L^{-1}), monopotassium phosphate (KH_2PO_4) (0.25 g L^{-1}), peptone (1.15 g L^{-1}) and yeast extract (2.5 g L^{-1}). All chemicals were purchased from local companies with 100% purity and for molecular biology grade. The pH of the prepared medium was adjusted to 5.0 and sterilised in an autoclave (model 50HV Hirayama) at 121°C for 15 min (Keturah *et al.*, 2014).

Methods

In this study, raw POME was firstly enzymatically hydrolysed using cellulase enzyme in order to release fermentable sugars as a carbon source for the microbe's growth. One-factor-at-time (OFAT) method was used to determine the optimal conditions in producing fermentable sugars via POME hydrolysis. NaOH was also added prior to POME hydrolysis to condition the substrate's pH as well as to enhance the fermentable sugar production. Then, the cultivation of *S. cerevisiae* was performed in various POME to basal medium ratios that are 0:100, 50:50 and 100:0. Moreover, the biomass yield, protein content and COD reduction were determined to identify the most suitable POME medium for maximum microbial protein production and pollutant removal. Analytical measurements were performed based on DOE standard method and APHA method. The overall process flow for MRE utilisation for microbial biomass protein production is illustrated in *Figure 1*.

Compositional Analysis of Mixed Raw Effluent (MRE)

A total of 200 mL sludge POME was filtered using cellulose filter (Whatman Filter No. 1) and put in a thimble and dried overnight in an oven. The dried solid MRE was then subjected to Soxhlet extraction with 130 mL petroleum ether for 4 hr to remove residual oil and extracts. The solid residue was then dried overnight at 60°C to remove traces of the solvent. The dried solid residue was then analysed for hemicellulose, cellulose and lignin as described by Ayeni *et al.* (2015). The cellulose content (% w/w) was calculated, assuming that oil and extractives, hemicellulose, lignin and cellulose were the only solid components in the entire mixed raw POME. The calculation is shown in Equation (1).

Cellulose (% w/w): 100% - hemicellulose - lignin - (oil + extractives) Equation (1)

Enzymatic Hydrolysis for MRE Pre-treatments

A volume of 100 mL MRE samples were adjusted to pH 4.7 (using 0.1 M citrate buffer or 0.1 M NaOH), followed by the addition of 1 mL cellulase enzyme. The samples were then incubated at 50°C with 150 rpm rotation for 24 hr (Noorshamsiana *et al.*, 2013). After the incubation process, the POME hydrolysate mixture was then sterilised at 121°C for 15 min. Parameters for the incubation were varied as follows: Enzyme dosages (1% to 5% v/v), temperatures (45°C-55°C), agitation rate (100-200 rpm) and pH (4.0-6.0).

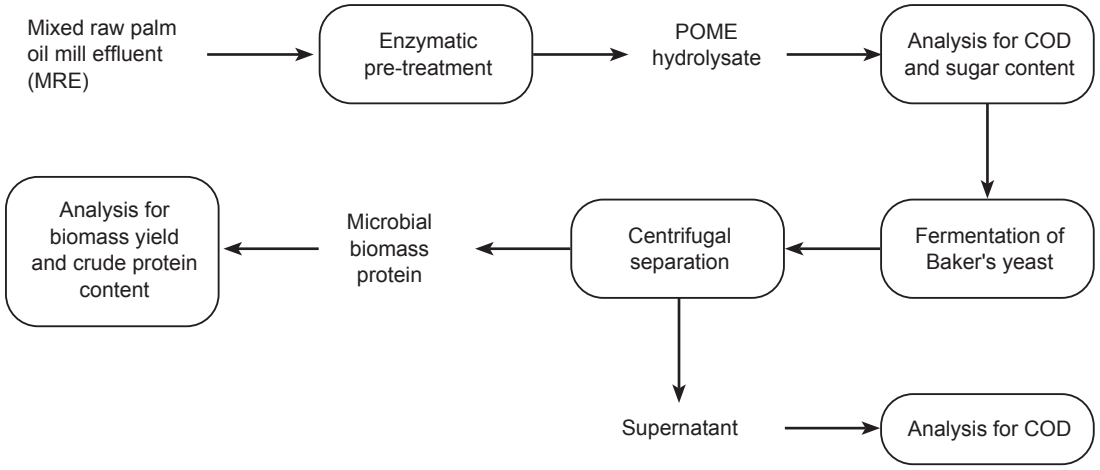

Figure 1. Process flow for mixed raw palm oil mill effluent (POME) utilisation for microbial biomass protein production.

Isolation of Baker's Yeast into Pure Culture

An amount of 1 g dry yeast was stirred in 100 mL distilled water in sterile condition inside laminar flow cabinet to prevent contamination. After 10-fold serial dilutions, 0.1 mL of the suspension was spread onto the surface of malt extract agar plates. Visible individual yeast colony was transferred onto new agar plates for seed culturing. All yeast cultures were incubated at 30°C for 48 hr.

Yeast Cultivation in MRE

One full loop of pure single colony from the seed culture was inoculated into 10 mL growth medium (GM) in a 50 mL Falcon tube overnight to promote yeast growth. The production of crude SCP was performed in a 250 mL flask using 100 mL POME medium or GM. The flask was inoculated with 10% (v/v) of the overnight seed culture and incubated at 30°C with agitation at 150 rpm for seven days using a rotary incubator shaker (MaxQ Mini 4450, Thermo Fisher Scientific, USA). After seven days, the fermentation was halted and the biomass was harvested. The resulting yeast biomass was subjected to centrifugal separation prior to the protein content analysis. The residual supernatant was collected for COD measurement.

The effect of medium composition for fermentation was studied by varying the POME to GM ratio. Two types of POME medium, *i.e.*, POME hydrolysate and untreated MRE were used in the fermentation medium preparation. The compositions of fermentation medium are shown in *Table 1*.

Chemical Oxygen Demand (COD) Analysis

Samples were diluted 10 times with distilled water for COD analysis. A volume of 0.2 mL diluted samples were transferred into the High Range Plus COD reagent vial (HACH, COD range 1500-15 000 ppm). The mixtures of samples and reagents were heated in the reactor at 150°C for 2 hr and then cooled to room temperature. The COD was then measured using a HACH DR890 colorimeter (APHA, 2018).

Reducing Sugar and Glucose Content

The reducing sugar content was measured by dinitrosalicylic acid (DNS) method (Miller, 1959). Approximately 1.5 mL sample was centrifuged at 5000 rpm for 20 min and 1 mL supernatant was decanted into a boiling tube. After adding 2 mL DNS reagent, the solution was then boiled in a water bath for 10 min. Then, the solution was made up to 10 mL with distilled water. The absorbance of the sample solution was measured at 540 nm using a UV-Visible spectrophotometer. The DNS calibration curve was developed using glucose standard concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 g L^{-1}.

Biomass Concentration

The biomass was collected by centrifuging the culture medium at 5000 rpm for 20 min. The supernatant was discarded and the pellet was washed several times with distilled water and then dried in an oven at 80°C for 24 hr (Keturah *et al.*, 2014). The biomass concentration or biomass yield was quantified in g L^{-1}.

TABLE 1. COMPOSITIONS OF FERMENTATION MEDIUM FOR SINGLE CELL PROTEIN PRODUCTION

Composition	The ratio of POME: Growth medium			Composition of nutrient in fermentation medium (g in 100 mL)				
	Untreated MRE (mL)	POME hydrolysate (mL)	Growth medium (mL)	Glucose	Yeast extract	Peptone	KH_2PO_4	$MgSO_4$
Reference Control 1 (CHP)		100	100	5	0.3	0.5	0.1	0.05
Control 2 (CP)	100							
1		50	50	2	0.3	0.5	0.1	0.05
2		100						
3		100		2	0.3	0.5	0.1	0.05
4	50		50	2	0.3	0.5	0.1	0.05
5	100							
6	100			2	0.3	0.5	0.1	0.05

Note: POME - palm oil mill effluent; MRE - mixed raw effluent; CP - control sample for untreated MRE; CHP - control sample for POME hydrolysate.

Crude Protein

Protein content (%) was determined by total nitrogen (TN) method with traditional conversion factor of 6.25 to convert N into protein content (Maehre *et al.*, 2018). The TN analysis was conducted using Dumas combustion method (Durmatherm, Gerhardt) with reactor temperature at 980°C. During the determination of TN, oxygen was used for combustion while helium was used as the carrier gas. This method was selected due to shorter analysis times, ease of operation and improved safety compared to the Kjeldahl method but with similar precision (Müller, 2017). The protein yield (g L^{-1}) was calculated by multiplying the biomass yield (g L^{-1}) and protein content (%) of the biomass.

RESULTS AND DISCUSSION

Compositional Analysis of MRE

The cellulose, hemicelluloses, lignin, oil content and moisture content were analysed prior to POME pre-treatment. Results obtained from the triplicate analysis revealed that the MRE contained water (90%-95%), solids (4%-7% dry weight), as well as oil and extractives (2%-3% dry weight). The lignocellulosic content of solid fraction in MRE was quantified. The solid MRE contained 44%-58% cellulose, 7%-11% hemicellulose, and 27%-43% lignin (*Table 2*). It is believed that high lignin content in POME contributed to the dark brownish POME colour together with tannin, humic acid and fulvic acid-like substance as well as phenolic compounds (Zahrim *et al.*, 2014). The cellulose amount was higher than the value reported by Khaw *et al.* (2008) whereas the hemicellulose content was comparable with Prasertsan *et al.* (2017). These findings may be due to the differences in sampling points, mills processes, and other factors (oil palm age and locality). Moreover, *Table 2* shows the organic content (measured as COD), total solids, crude protein, TN, reducing sugars, and glucose in MRE. The measured values obtained in this study were within the range when compared to previous studies. Apart from organic constituents, raw POME contains minerals and other nutrients such as phosphorus, potassium, calcium, and magnesium (Nahrul Hayawin *et al.*, 2017). The available cellulose and hemicellulose in POME can be converted into simple sugars and further utilised as fermentation media, while the available minerals and nutrients may be useful for microbial growth.

Pre-treatment of MRE for Fermentable Sugar Production

Polysaccharides in lignocellulosic materials are made from long homopolymer chain of glucose units connected by beta acetyl linkages. The low digestibility of lignocellulosic biomass for conversion into fermentable sugars is usually due to its lignin content, acetyl groups, and crystallinity. Therefore, physical and chemical pre-treatments followed by enzymatic hydrolysis are essential in order to improve the accessibility of the sugar components (Silvamany *et al.*, 2015). Enzymatic MRE pre-treatment using commercial cellulase enzymes were carried out. Optimisation of enzymatic hydrolysis was carried out using

TABLE 2. COMPOSITIONS OF MIXED RAW PALM OIL MILL EFFLUENT

Parameters	Unit	This study	Other studies
COD	mg L^{-1}	48 000-91 300	52 000-114 800
Moisture	%	90-95	95-96
Total solid	g L^{-1}	29-70	38-73
Total carbohydrate	%	NA	22.27-29.55
Crude protein	%	8.40-17.63	12.31-12.75
Total nitrogen	mg L^{-1}	536-1 295	420-8 540
Reducing sugar	g L^{-1}	5.29-14.79	1.53-12.45
Glucose	g L^{-1}	0-2.32	0.87-1.01
Cellulose	% dry weight	44-58	38-39
Hemicellulose	% dry weight	7-11	23-24
Lignin	% dry weight	27-43	22-26

Note: NA - not analysed.

Source: Kamal *et al.*, 2012; Khaw *et al.*, 2008; Nahrul Hayawin *et al.*, 2017; Nurul Adela *et al.*, 2016; Prasertsan *et al.*, 2017; Saifuddin and Refal, 2014.

OFAT method by varying one factor while keeping other parameters fixed in each experiment. In the first experiment, temperatures were varied at 45°C, 50°C and 55°C while pH, agitation, and enzyme dosage were fixed at 5.0, 150 rpm, and 1% (v/v), respectively. Fermentable sugar production measured as reducing sugar content was optimum at 50°C incubation temperature (*Figure 2*). The optimum temperature for the reaction and stability of three components, namely, endoglucanase, cellobiohydrolase, and beta-glucosidase in *Trichoderma* sp. is between 50°C and 60°C (Mun *et al.*, 2008). However, enzymatic hydrolysis activity becomes slower at 55°C and thus, producing less fermentable sugars.

In general, instead of using a buffer solution, MRE mixed with NaOH gave higher reducing sugar content. For instance, based on *Figure 2*, samples that were added with 100 mL 0.1 M of citrate buffer solution produced 13.14 g L^{-1} of fermentable sugars at incubation temperature of 50°C. In contrast, samples that were added with 100 mL 0.1 M NaOH produced about 34.78 g L^{-1} sugars. NaOH acts as a medium to adjust the pH which increases the amount of reducing sugars produced after the enzymatic hydrolysis. This finding is in agreement with Khaw *et al.* (2008) where lignin degradation by alkali will enhance the conversion of MRE into simple sugars. Alkali helps to promote hydrolysis and splitting of polymers into smaller molecules. During alkaline hydrolysis, the lignocellulosic compound swelled, leading to alteration of lignin structure and breaking of the ester and glycosidic chains (Noorshamsiana *et al.*, 2017).

Further experiments were carried out by treating the samples with 100 mL 0.1 M NaOH for pH adjustment. It was observed that the highest amount of fermentable sugars was produced when the pH of the substrate was at 4.7 (*Figure 3*). However, sugar production was not directly affected by the increment of enzyme concentration and agitation rate (*Figures 4* and *5*). Much lower enzyme loading was preferred in order to reduce the processing costs. Thus, the enzyme concentration of 1.0% (v/v) which gave comparable sugar production was used. Although the COD values were not affected by all parameters, however, COD values increased with the production of fermentable sugars. Based on OFAT method, the optimum parameters obtained for enzymatic hydrolysis of MRE using cellulase enzyme were pH 4.7, temperature 50°C, agitation rate 150 rpm, and enzyme dosage of 1 mL/100 mL MRE. MRE hydrolysate with reducing sugar content of about 41.63 g L^{-1} was produced whereas the COD value was 56 800 mg L^{-1}, compared to control that produced only 12.44 g L^{-1} of fermentable sugars (*Figure 4*). The fermentable sugars are utilised to complement glucose that is usually used in the preparation of conventional fermentation medium, due to the relatively high MRE hydrolysate reducing sugar content.

Yeast Growth Profile in Hydrolysed POME

The viable cell counts of the yeast isolate in the POME hydrolysate added with glucose, N source, and minerals are presented in *Table 3*. The cell count can only be visible after 72 hr cultivation. There was an intense growth of yeast after day 5 and day 6 of cultivation. However, after day 7, a reduction in viable cell count was recorded (4.00×10^8 CFU mL^{-1}). The mean viable cell counts of *S. cerevisiae* in POME hydrolysate were found to decrease after day 7. The reduction in growth can be attributed to limiting nutrients and oxygen, arising from the depletion of nutrients and oxygen in the media. Autolysis is enhanced by the exhaustion of nutrients and oxygen (Ojokoh and Uzeh, 2005). Thus, yeast cultivation will stop at day 7 in order to obtain maximum biomass yield from the fermentation.

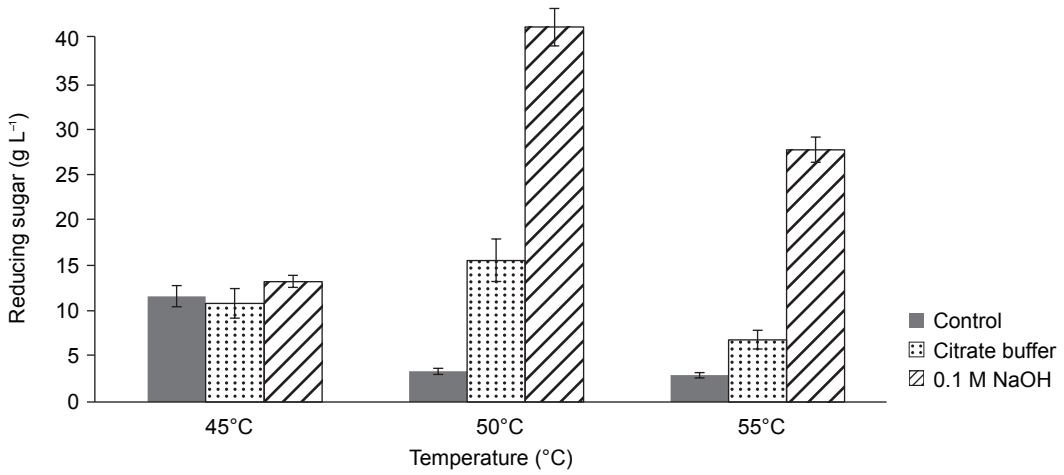

Figure 2. Effect of incubation temperature on reducing sugar concentration (enzyme dosage: 1 mL/ 100 mL substrate, pH: 4.7, agitation rate: 150 rpm).

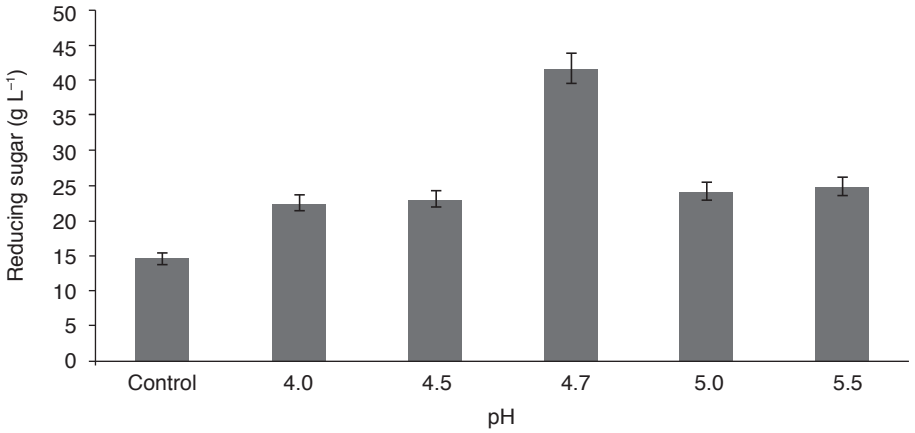

Figure 3. Effect of pH on fermentable reducing sugar production (enzyme dosage: 1 mL 100 mL substrate, agitation rate: 150 rpm, incubation temperature: 50°C).

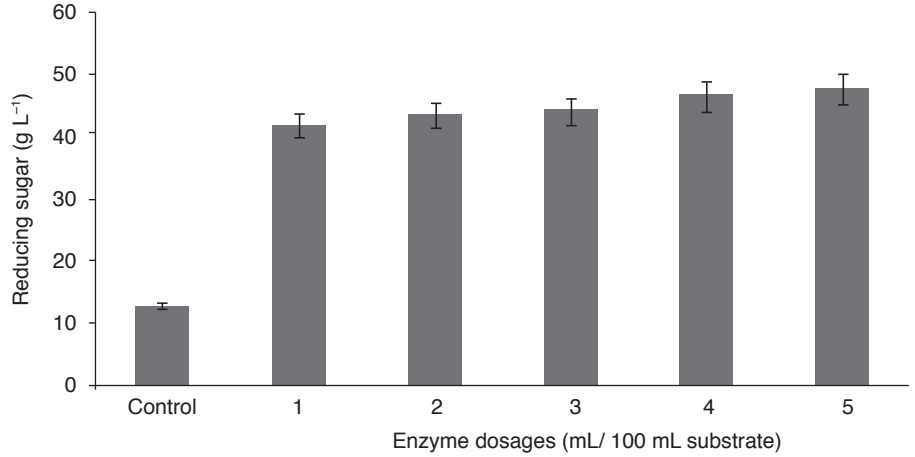

Figure 4. Effect of enzyme dosages on fermentable sugar production (pH: 4.7, agitation rate: 150 rpm, incubation temperature: 50°C).

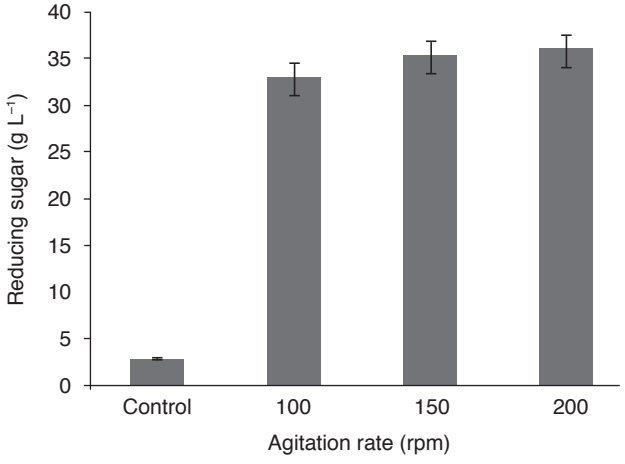

Figure 5. Effect of agitation rate on fermentable sugar production (enzyme dosage: 1 mL/ 100 mL substrate, pH: 4.7, incubation temperature: 50°C).

TABLE 3. VIABLE CELL COUNT OF *S. cerevisiae* IN POME HYDROLYSATE MEDIUM DURING 7-DAYS INCUBATION

Day	CFU mL^{-1}
1	Not visible
2	Not visible
3	5.00×10^7
4	8.00×10^7
5	2.04×10^8
6	1.03×10^9
7	4.00×10^8

Production of Microbial Biomass Protein with Different POME Compositions

After 7 days of fermentation, the growth of *S. cerevisiae* in control and experimental media were determined by quantifying the biomass yield. The biomass yield, COD reduction, and protein content obtained after yeast fermentation in different medium containing hydrolysed MRE (POME hydrolysate) or untreated POME as well as supplemented fermentation medium are tabulated in *Table 4*. Compositions 1 to 3 consisted of POME hydrolysate as fermentation medium supplemented with nutrients as described in the

TABLE 4. EFFECT OF DIFFERENT FERMENTATION MEDIUM ON BIOMASS YIELD, COD REDUCTION AND PROTEIN CONTENT

Composition No.	Media	Biomass yield (g L^{-1})	COD reduction (%)	Protein content (%)	Protein yield (g L^{-1})
Reference	100% GM	29.51 ± 0.35	22.63 ± 0.05	58.63 ± 0.95	17.30 ± 0.25
CHP	Control hydrolysed POME	8.34 ± 0.32	62.22 ± 2.50	11.05 ± 0.04	0.92 ± 0.03
CP	Control untreated MRE	29.93 ± 1.89	59.87 ± 1.47	17.23 ± 0.41	5.45 ± 0.44
1	50% HP + 50% GM	18.35 ± 1.21	55.39 ± 1.00	24.42 ± 0.11	4.52 ± 0.30
2	100% HP	17.66 ± 1.20	68.32 ± 2.50	18.72 ± 0.42	3.21 ± 0.20
3	HP + 2% glucose + YE + peptone + minerals	28.92 ± 1.37	79.12 ± 1.87	24.79 ± 0.33	7.34 ± 0.43
4	50% UP + 50% GM	26.64 ± 0.99	56.98 ± 2.30	18.92 ± 0.66	5.28 ± 0.34
5	100% UP	31.13 ± 3.00	62.85 ± 2.50	24.00 ± 1.96	6.78 ± 0.51
6	UP + 2% glucose + YE + peptone + minerals	51.66 ± 3.47	56.07 ± 2.70	18.09 ± 0.70	9.83 ± 0.79

Note: POME - palm oil mill effluent; COD - chemical oxygen demand; CP - control sample for untreated MRE; CHP - control sample for POME hydrolysate; HP - hydrolysed POME; UP - untreated MRE; YE - yeast extract; GM - growth medium.

method section. Composition 3 which contained 100 mL POME hydrolysate supplemented with 2 g glucose, 0.5 g peptone, 0.3 g yeast extract, 0.1 g KH_2PO_4, and 0.05 g magnesium sulphate ($MgSO_4$) produced the highest biomass yield and COD reduction at 26.74 g L^{-1} and 79.12%, respectively. The recorded density of yeast growth for this composition was 1.03×10^9 CFU mL^{-1}. This shows that the yeast cells have utilised more sugars and produced more biomass (Keturah *et al*., 2014). In contrast, the biomass yield, COD reduction, and protein content in control POME hydrolysate were 8.33 g L^{-1}, 62.22% and 11.05%, respectively. No colony was formed in aliquot spotted agar plate indicating that the biomass and protein determined in the POME hydrolysate control was not affected by the yeast growth. For composition 2 (100% hydrolysed POME), the growth of yeast was observed in the medium but with lower biomass yield, since no glucose and nutrients were added into this medium. This indicated that the yeast growth is highly dependent on limited fermentable sugars available in POME hydrolysate with reducing sugar content of approximately 4 g/100 mL. Previous study showed that lower yeast growth was observed when the fermentation media (papaya extract juice) were diluted with 400 mL and 600 mL sterile water as compared by using undiluted juice as the fermentation medium. The poor cell growth in diluted fermentation substrate may be attributed to reduction of available sugars and in the concentration of growth factors (Ojokoh and Uzeh, 2005).

Composition 6 which contained 100 mL untreated POME supplemented with 2 g glucose, 0.3 g yeast extract, 0.5 g peptone, 0.1 g KH_2PO_4, and 0.05 g $MgSO_4$ produced the highest biomass yield (51.66 g L^{-1}). However, the high biomass yield recorded may be contributed by the initial suspended solids that existed in the untreated POME samples. This can be seen from the raw POME control samples (29.93 g L^{-1} solid biomass). COD reduction during fermentation was relatively lower at 56.07%. The growth of yeast was observed in this medium with the addition of N source (yeast extract and peptone) together with carbon source (existing COD) in the POME samples. COD reduction of 62.85% in medium 5 (100% untreated POME) was highest among untreated POME samples. Since no additional carbon sources and nutrients were added into this medium, the yeast utilised any carbon, N and micronutrient sources that were readily available in the untreated POME samples. The yeast (*S. cerevisiae*) may also be able to hydrolyse complex carbohydrate in untreated POME into sugars that serve as carbon source in synthesising microbial biomass during the fermentation process (Aruna *et al*., 2017).

The biomass produced after fermentation was analysed for protein content. The initial protein content in untreated POME and POME hydrolysate were 17.26% and 11.05%, respectively. The highest protein content was obtained in composition 3 (24.79%). With this amount, the resulting biomass is quite suitable for animal feed use (Iwuagwu and Ugwuanyi, 2014). The protein yield in composition 3 was the highest (7.34 g L^{-1}) among POME hydrolysate medium due to its highest biomass yield. Protein yield in untreated POME was higher (11.8 g L^{-1}) than POME hydrolysate, but with lower protein increment (5.45 g L^{-1}) compared to initial protein content prior to yeast fermentation. However, the biomass yield was comparable to GM although the protein content was much lower. This is due to 5% glucose was used as carbon source in GM whereas

only 2% glucose was added with 4% reducing sugar (mixed of glucose and pentose) in medium 3. Earlier study stated that *S. cerevisiae* preferentially utilises glucose as the source of carbon. However, following its depletion, it can utilise a wide variety of other carbons including non-fermentable compounds (Turcotte *et al.*, 2009).

Table 5 shows that medium compositions affect the increment of biomass and protein yield after fermentation. The use of 100% POME hydrolysate supplemented with inorganic carbon and N source as well as micronutrient in fermentation medium (composition 3) gave substantial increase in biomass and protein yield of more than 3.44 and 7.74 folds, respectively. The crude protein content in that particular sample was 24.79%. Nevertheless, composition 6 recorded the highest biomass and protein yield of 51.66 and 9.83 g L^{-1}, respectively. The biomass and protein yield increment after yeast fermentation were only 1.71 and 1.89 folds, respectively. These findings demonstrated similar trend with a study by Aruna *et al.* (2017) in which fermented yam peels with *S. cerevisae* supplemented with N source produced 15.54% crude protein as compared to unfermented yam peels and non-supplemented *S. cerevisae* fermented yam peels that produced 6.60% and 11.08% crude protein, respectively. In addition, COD reduction was not influenced by composition of the fermentation medium.

Compared to other studies presented in *Table 6*, this study managed to produce higher biomass but with lower protein content. This is because most of the biomass was in the form of suspended solid (readily present in the raw material). However, yeast fermentation seemed to significantly increase the protein content in POME hydrolysate from 11% to 29%. For untreated POME substrate, the protein increment was from 17% to 24%. The COD reduction of 79.12% was comparable to Barker and

TABLE 5. INCREMENT OF BIOMASS AND PROTEIN YIELD AFTER YEAST FERMENTATION IN HYDROLYSED POME

Samples	Media	Biomass yield increment (fold)	Protein yield increment (fold)
1	50% HP + 50% GM	2.20 ± 0.72	4.90 ± 1.62
2	100% HP	2.02 ± 0.34	3.34 ± 0.44
3	HP + 2% glucose + YE + peptone + minerals	3.44 ± 0.41	7.74 ± 1.07
4	50% UP + 50% GM	0.97 ± 0.16	1.05 ± 0.19
5	100% UP	0.94 ± 0.16	1.28 ± 0.17
6	UP + 2% glucose + YE + peptone + minerals	1.71 ± 0.13	1.89 ± 0.51

Note: POME - palm oil mill effluent; COD - chemical oxygen demand; CP - control sample for untreated mixed raw POME (MRE); CHP - control sample for POME hydrolysate; UP - untreated MRE; HP - hydrolysed POME; YE - yeast extract; GM - growth medium.

TABLE 6. COMPARISONS ON YEAST AND FUNGAL BIOMASS PRODUCTIONS FROM DIFFERENT SUBSTRATES

Substrate/ Microorganism	Biomass yield (g L^{-1})	COD reduction (%)	Protein content (%)	Protein yield (g L^{-1})	References
Untreated POME/ *S. cerevisiae*	63.84 ± 6.01	59.02 ± 5.34	18.09 ± 2.81	11.80 ± 1.55	This study
POME hydrolysate/ *S. cerevisiae*	28.92 ± 3.43	79.12 ± 3.73	24.79 ± 1.31	7.34 ± 1.06	This study
POME/ *Saccharomyces* sp. L3	4.42	83.00	27.00	1.19	Iwuagwu and Ugwuanyi (2014)
Pineapple waste/ *S. cerevisiae*	5.71	n/a	n/a	2.76	Dhanasekaran *et al.* (2011)
Cabbage juice/ *S. cerevisiae*	8.00	n/a	35.00	2.00	Choi and Park (2003)
Vegetable waste hydrolysate/*S. cerevisiae*	8.10	n/a	41.30	3.35	Stabnikova *et al.* (2005)
Potato wastewater/ *Candida utilis*	33.25	n/a	36.70	12.20	Kurcz *et al.* (2018)
Steriliser condensate/ Isolated cellulolytic fungi	8.30	n/a	30.30	2.48	Cheah and Leslie (1987)

Note: n/a - not available; POME - palm oil mill effluent; COD - chemical oxygen demand.

Worgan (1981) with a reduction of 77% COD during mycoprotein production from POME using *Yarrowia lipolytica* NCIM 3589 culture. On the other hand, COD reduction was slightly lower when compared to the use of POME for bioethanol and biogas production at 91% and 82%, respectively (Alam *et al*., 2009; Prasertsan *et al*., 2017).

CONCLUSION

POME hydrolysate was utilised as medium for the production of microbial biomass protein, that is also known as crude SCP. Fermentation of mixed raw POME either in raw or hydrolysed form, using *S. cerevisiae* with and without the addition of carbon and N sources resulted in microbial protein production. However, higher yield was obtained when fermentation of the *S. cerevisiae* was carried out using undiluted POME hydrolysate as the substrate. The fermentation produced 28.92 g L^{-1} biomass with 24.79% protein content and consequently reduced organic pollutant up to 79.12% COD reduction. The yeast fermentation in undiluted hydrolysed POME supplemented with additional carbon and N sources recorded the highest increase in biomass and protein contents that of 3.44 and 7.74 folds, respectively. Considering these significant enhanced protein contents in the POME, it is a potential protein supplement in animal diet. Besides this, scale-up studies should be conducted in order to develop the present process, which would offer a low-cost and abundant substrate for SCP production.

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REFERENCES

Alam, M Z; Kabbashi, N A and Hussin, S N I S (2009). Production of bioethanol by direct bioconversion of oil-palm industrial effluent in a stirred-tank bioreactor. *J. Ind. Microbiol Biotechnol., 36*: 801-808.

Ali Amat, N A; Tan, Y H; Lau, W J; Lai, G S; Ong, C S; Mokhtar, N M; Sani, N A A; Ismail, A F; Goh, P S; Chong, K C and Lai, S O (2015). Tackling colour issue of anaerobically-treated palm oil mill effluent using membrane technology. *J. Water Process Eng., 8*: 221-226.

Anupama and Ravindra, P (2000). Value-added food: Single cell protein. *Biotechnol. Adv., 18(6)*: 459-479.

APHA (2018). 5220 Chemical Oxygen Demand (COD). In Standard Methods for the Examination of Water and Wastewater. American Public Health Association.

Aruna, T E; Aworh, O C; Raji, A O and Olagunju, A I (2017). Protein enrichment of yam peels by fermentation with *Saccharomyces cerevisiae* (BY4743). *Ann. Agric. Sci., 62(1)*: 33-37.

Ayeni, A O; Adeeyo, O A; Oresegun, O M and Oladimeji, T E (2015). Compositional analysis of lignocellulosic materials: Evaluation of an economically viable method suitable for woody and non-woody biomass. *Am. J. Eng. Res., 4(4)*: 14-19.

Barker, T W and Worgan, J T (1981). The utilisation of palm oil processing effluents as substrates for microbial protein production by the fungus *Aspergillus oryzae*. *European J. Appl. Microbiol. Biotechnol., 11(4)*: 234-240.

Cheah, S C and Leslie, O C (1987). Development of a process for palm oil mill steriliser condensate utilisation. *Proc. of the National Symposium of Oil Palm By-product for Agro-based Industries*. p. 203-220.

Chia, W Y; Chong, Y Y; Chew, K W; Vimali, E; Jayaram, M; Selvarajoo, A; Muthuvelu, K S; Varalakshmi, P; Show, P L and Arumugasamy, S K (2020). Outlook on biorefinery potential of palm oil mill effluent for resource recovery. *J. Environ. Chem. Eng., 8(6)*: 104519.

Choi, M H and Park, Y H (2003). Production of yeast biomass using waste Chinese cabbage. *Biomass Bioenergy, 25*: 221-226.

Dhanasekaran, D; Lawanya, S; Saha, S; Thajuddin, N and Panneerselvam, A (2011). Production of single cell protein from pineapple waste using yeast. *Innov. Rom. Food Biotechnol., 8*: 26-32.

Iwuagwu, J O and Ugwuanyi, J O (2014). Treatment and valorization of palm oil mill effluent through production of food grade yeast biomass. *J. Waste Manag., 2014*: 1-9.

Izah, S C (2018). Feed potentials of *Saccharomyces cerevisiae* biomass cultivated in palm oil and cassava mill effluents. *J. Bacteriol. Mycol. Open Access, 6(5)*: 287-293.

Izzi, A Z; Yusuf, N N A N; Ishak, W F W and Kim, H (2020). Box-Behnken-Design based optimization strategy for alkaline pretreatment of palm oil mill

effluent for producing reducing sugar. *IOP Conf. Ser.: Earth Environ. Sci., 596(1)*.

Kamal, S A; Md Jahim, J; Anuar, N; Hassan, O; Wan Daud, W R; Mansor, M F and Rashid, S S (2012). Pre-treatment effect of palm oil mill effluent (POME) during hydrogen production by a local isolate *Clostridium butyricum*. *Int. J. Adv. Sci. Eng. Inf. Technol., 2*: 325-331.

Kamyab, H; Din, M F M; Keyvanfar, A; Majid, M Z A; Talaiekhozani, A; Shafaghat, A; Lee, C T; Shiun, L J and Ismail, H H (2015). Efficiency of microalgae chlamydomonas on the removal of pollutants from palm oil mill effluent (POME). *Energy Procedia, 75*: 2400-2408.

Keturah, I; Sandrasegarampillai, B and Vasanthy, A (2014). Baker's yeast biomass production with rice as carbon and soy meal as nitrogen sources. *Malays. J. Microbiol., 10(3)*: 205-214.

Khaw, T S; Hassan, M A and Ariff, A (2008). Enzymatic saccharification of pretreated solid palm oil mill effluent and oil palm fruit fiber. *Pertanika J. Sci. Technol., 16(2)*: 157-169.

Kurcz, A; Blazejak, S; Kot, A M; Bzducha-Wrobel, A and Kieliszek, M (2018). Application of industrial wastes for the production of microbial single-cell protein by fodder yeast *Candida utilis*. *Waste Biomass Valor., 9*: 57-64.

Kushairi, A; Ong-Abdullah, M; Nambiappan, B; Hishamuddin, E; Bidin, M N I Z; Ghazali, R; Subramaniam, V; Sundram, S and Parveez, G K A (2019). Oil palm economic performance in Malaysia and R&D progress in 2018. *J. Oil Palm Res., 31(2)*: 165-194.

Ma, A N (1999). Treatment of palm oil mill effluent. *Oil Palm and the Environment: A Malaysian Perspective* (Singh, G; Lim, K H; Leng, T and David, L K eds.). Malaysian Oil Palm Growers' Council. Kuala Lumpur. p. 113-126.

Maehre, H K; Dalhein, L; Edvinsen, G K; Elvevoll, E O and Jensen, I-J (2018). Protein determination - Method matters. *Foods (Basel, Switzerland), 7(1)*: 5. DOI: 10.3390/foods7010005.

Miller, G L (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem., 31(3)*: 426-428.

Müller, J (2017). Dumas or Kjeldahl for reference analysis? Comparison and considerations for nitrogen/protein analysis of food and feed. *Anal. Beyond Meas., (June):* 1-5.

Mun, W K; Rahman, N A; Abd-Aziz, S; Sabaratnam, V and Hassan, M A (2008). Enzymatic hydrolysis of palm oil mill effluent solid using mixed cellulases from locally isolated fungi. *Res. J. Microbiol., 3(6)*: 474-481.

Nahrul Hayawin, Z; Nor Faizah, J; Ropandi, M and Astimar, A A (2017). A review on the development of palm oil mill effluent (POME) final discharge polishing treatments. *J. Oil Palm Res., 29(4)*: 528-540.

Nasrullah, M; Singh, L; Krishnan, S; Sakinah, M and Zularisam, A W (2018). Electrode design for electrochemical cell to treat palm oil mill effluent by electrocoagulation process. *Environ. Technol. Innov., 9*: 323-341.

Noorshamsiana, A W; Nur Eliyanti, A; Fatiha, I and Astimar, A A (2017). A review on extraction processes of lignocellulosic chemicals from oil palm biomass. *J. Oil Palm Res., 29(4)*: 512-527.

Noorshamsiana, A W; Astimar, A A; Nor Hayati, M; Nor Faizah, J; Mohamadiah, B and Norhayati, S (2013). Optimisation of enzymatic sludge palm oil recovery from palm oil mill effluent using response surface methodology. *J. Oil Palm Res., 25(3)*: 348-356.

Numan, A H; Yahaya, H; Ngatiman, M; Nor Faizah, J; Noorshamsiana, A W and Rohaya, M H (2019). Assessment of the effluent polishing plant using a ultrafiltration membrane installed at a palm oil mill. *Environ. Prot. Eng., 45(1)*: 155-170.

Nurul Adela, B; Nasrin, A B and Loh, S K (2016). Palm oil mill effluent as a low - cost substrate for bioflocculant production by *Bacillus marisflavi* NA8. *Bioresour. Bioprocess., 3*: 2-8.

Nurul Adela, B; Loh, S K; Nasrin, A B and Jahim, J M (2020). Enzymatic hydrolysate of palm oil mill effluent as potential substrate for bioflocculant BM-8 production. *Waste Biomass Valor., 11(1)*: 17-29.

Ojokoh, A O and Uzeh, R E (2005). Production of *Saccharomyces cerevisiae* biomass in papaya extract medium. *Afr. J. Biotechnol., 4(11)*: 1281-1284.

Parthasarathy, S; Mohammed, R R; Chong, M F; Gomes, R L and Manickam, S (2016). A novel hybrid approach of activated carbon and ultrasound cavitation for the intensification of palm oil mill effluent (POME) polishing. *J. Clean. Prod., 112*: 1218-1226.

Patelski, P; Berłowska, J; Balcerek, M; Dziekońska-Kubczak, U; Pielech-Przybylska, K; Dygas, D

and Jedrasik, J (2020). Conversion of potato industrywaste into fodder yeast biomass. *Processes, 8(4)*: 453.

Prasertsan, P; Khangkhachit, W; Duangsuwan, W; Mamimin, C and O-Thong, S (2017). Direct hydrolysis of palm oil mill effluent by xylanase enzyme to enhance biogas production using two-steps thermophilic fermentation under non-sterile condition. *Int. J. Hydrog. Energy, 42(45)*: 27759-27766.

Saifuddin, N and Refal, H (2014). Microwave assisted alkaline pretreatment and microwave assisted enzymatic hydrolysis of palm oil mill effluent (POME) for optimum fermentable sugar yield. *Chem. Sci. Trans., 3*: 350-358.

Sharif, M; Zafar, M H; Aqib, A I; Saeed, M; Farag, M R and Alagawany, M (2021). Single cell protein: Sources, mechanism of production, nutritional value and its uses in aquaculture nutrition. *Aquaculture, 531*: 735885.

Silvamany, H; Harun, S; Mumtaz, T and Jahim, J M (2015). Recovery of fermentable sugars from palm oil mill effluent via enzymatic hydrolysis. *Jurnal Teknologi, 77(33)*: 115-121.

Spalvins, K; Ivanovs, K and Blumberga, D (2018). Single cell protein production from waste biomass: Review of various agricultural by-products. *Agron. Res., 16*: 1493-1508.

Stabnikova, O; Wang, J; Ding, H B and Tay, J (2005). Biotransformation of vegetable and fruit processing wastes into yeast biomass enriched with selenium. *Bioresour. Technol., 96*: 747-751.

Turcotte, B; Liang, X B; Robert, F and Soontorngun, N (2009). Transcriptional regulation of nonfermentable carbon utilization in budding yeast. *FEMS Yeast Res., 10*: 2-13.

Zahrim, A Y; Nasimah, A and Hilal, N (2014). Pollutants analysis during conventional palm oil mill effluent (POME) ponding system and decolourisation of anaerobically treated POME via calcium lactate-polyacrylamide. *J. Water Process Eng., 4*: 159-165.

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PALM OLEIN LUBRICATES AND REMOVES FRESHLY BAKED CONCRETE FROM ITS FORMWORK

SOH KHEANG LOH[1]* and NGATIMAN MUZZAMMIL[1]

ABSTRACT

Typical use of toxic and flammable petroleum-based mould oil for concrete manufacturing and its ever-increasing price have prompted the search for a user-friendlier vegetable oil alternative. This study examines the potential use of a refined palm oil, i.e.*, palm olein for mould release lubrication. Its use eliminates additional steps required for structural modification and avoids food insecurity conflict due to its abundance and versatility. Blending palm olein (base oil) with a tackifier (specialty additive) was attempted to investigate their mould release characteristics. The concerned lubricating properties,* i.e.*, viscosity index and flash point of the formulated mould oil were comparable to that of petroleum counterpart, while those distinguishable characteristics were saponifiable value, acid number and free fatty acid, which contributed to better mould release ability. The unmodified palm olein-based mould oil exhibited moderate level of oxidative stability with added advantages of non-corrosiveness and biodegradability. The optimised blend (90:10 of oil: additive) on trial could detach the moulded concrete from its formwork smoothly, showing good releasing ability desirable for concrete making.*

Keywords: interfacial tension, mould release ability, palm olein, tackifier.

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INTRODUCTION

The global lubricant market has undergone dramatic changes due to overall flat demand, sharp shifts in consumption, increased competition and greater pressure on profits (Sahara Business Group, 2021). In 2019, the world demand for lubricants reached 36.8 million tonnes (Sönnichsen, 2021). While the growth in Asia Pacific and the Middle East was due to massive economic development and rise in the industrial sector, other mature markets, for example, the USA, Europe and Japan have remained flat since 1991 (Research and Markets, 2019; Theodori *et al.*, 2004) due to stricter environmental regulations on lubricant use and disposal plus progressive introduction of synthetic oil with longer drain intervals. Many new lubricants are "filled for life" unlike their conventional counterparts, *i.e.*, they can be recycled and reused while some of them are able to withstand long operation hours (EuropaLub, 2015). Although global share of vegetable oil-based lubricants or biolubricants market in the 1990s was very small, for example, only ~2% was consumed in Europe from its total 10.2 million tonnes market (Rac and Vencl, 2008), the global demand is anticipated to grow at ~7% annual rate to reach 1.12 million tonnes by 2024 in responding to various environmental issues associated with conventional lubricants (Grand View Research, 2016).

Biolubricants have gained much share nowadays for the desirable environmental features, for example, rapid biodegradability and low toxicity (Hassan *et al.*, 2016) but their use is refrained by high cost (Ho *et al.*, 2019). An ideal total replacement of conventional lubricants would only be anticipated industrially, if biolubricants could ever meet tough performance requirements and be low-cost (Woma

[1] Malaysian Palm Oil Board,
6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.

* *Corresponding author e-mail: lohsk@mpob.gov.my*

et al., 2019). To date, biolubricants are about 1.5 to 5.0 times more expensive than the fossil-based counterparts (Srivastava and Sahai, 2013; Theodori *et al.*, 2004). Price factor is indeed crucial for market penetration although other environmental factors are also important. Two significant market opportunities for biolubricants exist; sectors with high probability of accidental exposure of lubricant to sensitive environment and total loss of lubricants to the atmosphere, considering that they are low in ecotoxicity with high biodegradability (Mobarak *et al.*, 2014; Srivastava and Sahai, 2013). Typical example of a total loss lubricant which ends up virtually entirely in the environment during normal use is mould release agent (Theodori *et al.*, 2004). Its mould release ability is highly required when applied onto surfaces of building materials (such as concrete, steel, cement, *etc.*) during manufacturing.

The demand for building materials in construction industry is indeed huge due to vast development especially in developing and densely populated countries. In particular, during concrete block production, a mould release agent, normally fossil-based, is frequently used to perform lubrication during detachment of the moulded concrete from the formwork. Its use creates an unpleasant workplace and causes health problem, as it is flammable and toxic in nature (Libessart *et al.*, 2014). The soaring fuel costs, plus an increased workers' awareness on healthy workplace have driven higher demand for environmental-friendly alternatives with better quality and regulatory conformity. Vegetable oil-based mould release lubricants stand a chance as they are not only non-toxic, renewable and biodegradable, also perform flexible surface activity and release ability dependent on polarity of the fatty acids present (Loh and Choo, 2012).

Mould release agents have been manufactured from mineral oils and waxes in the past with the incorporation of a chemically-active compound such as oleic acid to perform good release property. Others have co-existed with vegetable oil, aliphatic carboxylic acid ester, nonionic and anionic surfactant or antioxidant and polyacrylate forming oil-in-water emulsion (Nielsen, 1985; Wittich *et al.*, 1995) to ease release of a moulded concrete piece from the mould. A more advanced mould oil was also developed using industrial wastes such as used cooking oil and engine oil (Yi, 1996). Non-refined vegetable oil such as coconut oil, corn oil, palm oil, cottonseed oil, rapeseed oil, soy oil and sunflower oil, either as a mixture or used individually with an emulsifier, to serve as a mould release agent with sealing effect to prevent the passage of water into the concrete (Lightcap, 1997). As a matter of fact, bio-based mould oils are less-performed than the fossil-based counterparts due to the feedstock natural characteristics. The inferior mould release property such as oxidation resistance, corrosion resistance, viscosity, tackiness, stability and anti-wear can be overcome by incorporating properly designed specialty additives to suit for the intended use. Commercially available vegetable oil-based mould release agents mixed with oleic acid, alcohol and mineral seal oil (viscosity reducer) could reduce adhesion between concrete and formwork, thus, improving the life of the forms (LaFay and Neltner, 2002). The concrete-formwork lubricated with vegetable oil or mixed oil films shows overall satisfactory quality facings and release performance (Libessart *et al.*, 2014). All the improved mould release agents invented so far exhibit a chemical rather than a barrier (physical) release. This means that vegetable oil-based mould release agents produce thin and harmless soap, up to double layers, between the concrete's surface and the mould itself in producing not just a smooth finish to the concrete but also a hydrophobic surface resistant to weather attack (Libessart *et al.*, 2014). Their use ensures lesser volume consumed than the traditional agents, offers protection of steel mould casings from corrosion and extends formwork lifespan.

Issue on incidental contact of lubricant containing mineral oil saturated hydrocarbon (MOSH) and mineral oil aromatic hydrocarbon (MOAH) in crushing and refining plants during food processing has received much attention in the 21st century due to an increased awareness and modernisation. Several requirements such as Hazard Analysis Critical Control Point (HACCP) and *'Makanan Selamat Tanggungjawab Industri'* (MeSTI) certification schemes (KKM, 2018) have been implemented to monitor, certify and ensure food safety and hygiene for human consumption. Foodstuff can be unintentionally contaminated during transportation/processing, via environmental sources, or through migration from food contact materials (Pirow *et al.*, 2019). Mineral oil-based lubricants for food machinery uses will cause incidental food contact while those intended for external non-food applications include, for example, mould release agents, that will pose potential health risk to workers through inhalation exposure, in long run. Mineral oil-based release agents are also used in production processes, and thus, might as well cause food contamination. Human health threats such as toxicity and carcinogenicity (Pirow *et al.*, 2019) due to exposure of mineral oil at a risky workplace, for example, concrete mould factory, has also triggered considerable attention worthy of investigation. Palm oil can be an alternative to provide high cleanliness and less dermatological problem at the workplace due to its promising skin compatibility (Dandan and Samion, 2017).

Undeniably, use of edible oil as lubricant, in general, faces pressure on food shortage crisis, food *vs.* fuel debate, energy security, and high oil price and

dependence. In a global context, however, oil palm presents a huge opportunity via biotechnological advancement in terms of oil yield and productivity. To date, it is the highest world average oil bearing crop with yield at ± 3.5 t ha^{-1} yr^{-1} (Singh *et al.*, 2021) compared to other crops, such as rapeseed (0.75 t ha^{-1} yr^{-1}), soybean (0.56 t ha^{-1} yr^{-1}) and coconut (0.3 t ha^{-1} yr^{-1}) (Woittiez *et al.*, 2017). This implies that oil palm is up to 10 times more productive per unit land area, and coupled with advanced and modern biotechnology approaches, shows evidence of future oil yield escalation and enhancement (Singh *et al.*, 2021) to accommodate majorly food and sparsely (remaining) for non-food applications. Its high productivity ensures replenishable feedstock that will be able to meet the demand for vegetable-based lubricants in the future (Syahrullail *et al.*, 2011). Relevant research literature on tribological performance has shown that vegetable oil-based lubricants are convincing to substitute mineral oils (Chan *et al.*, 2018; Ho *et al.*, 2019; Rao *et al.*, 2018) with added environmental and social benefits. As such, the use of palm oil for non-food applications, *i.e.*, biolubricant, as small as it seems, is aligned with the United Nations Sustainable Development Goals (SDG) No. 8, 9 and 12 in promoting productive employment and decent work, fostering innovation and ensuring sustainable production pattern. With an increased environmental concerns and technological advancement, the future of lubricant industry will shift towards producing highly specialised green lubricants from renewable sources such as vegetable oil, waste oil, animal fats and other biomass feedstocks (Shah *et al.*, 2020). Next, many standard lubricants currently in use will be replaced with highly functionalised specialty counterparts, to be derived from tailor-made renewable resources, in addressing growing demand for ecotoxicological as well as sustainability criteria. This research contributes a small yet important aspect to the whole palm oil value chain in making the SDGs part of the industry's routine for a low-carbon green economy.

In the European Union, vegetable oil-based concrete release agents are usually based on rapeseed and soy oils and their esters (Theodori *et al.*, 2004). Although vegetable oils are highly recommended, they have limited applicability as high-performance loss lubricant due to their inherently poorer lubrication properties (viscosity, thermal and oxidative stability and tackiness) compared to conventional or synthetic lubricants. The ability of pure vegetable oil to release a formed material under thermal stresses from its mould would be jeopardised, if it is not structurally modified or boosted with additives (Woma *et al.*, 2019). Comparatively, the use of non-edible plant oils, such as *Jatropha* and *Moringa* oils (Souza *et al.*, 2019) as pure and mixed blends (Farfan-Cabrera *et al.*, 2020) or synthetic form (Pindit *et al.*, 2021), can be considered but their availability is dependent on geographical locations and climate. Enzymatically and chemically catalysed high acid oil, a by-product of vegetable oil refining, such as soybean (Fernandes *et al.*, 2021) and palm fatty acid distillates (Fernandes *et al.*, 2018; Pindit *et al.*, 2021), plus structurally modified used/waste cooking oil (Borugadda and Goud, 2016; Hussein *et al.*, 2021; Owuna *et al.*, 2020) are also exploited as sources of environmental-friendly biolubricants. Even residual oil such as those remaining in spent bleaching earth after oil refining can be of potential (Kheang *et al.*, 2007; Sukirno and Farhandika, 2020). However, the production of these degraded waste oils, which have been loaded with impurities, into high-performance biolubricants will be costly as several steps such as pre-treatment, complex modification and post-treatment with specialty additives are required to improve their quality (Borugadda and Goud, 2016; Loh *et al.*, 2006; Zhang *et al.*, 2020). Furthermore, these oils are of limited supply and highly priced now as they are also much demanded as a biodiesel feedstock elsewhere (Argus Media, 2021). With inherently acceptable lubricity property and corrosion inhibition (Hassan *et al.*, 2016; Loh and Choo, 2012), palm olein can be a potential readily usable base fluid alternative to petroleum for making into a mould release lubricant. Palm olein, the refined liquid product from crude palm oil (CPO), fulfilling the cleanliness criteria in base stock selection (Woma *et al.*, 2019) with acceptable unsaturation level to resist oxidation (Loh and Choo, 2012) is thus, the right candidate amongst the ranges of palm oil products for mould oil formulation. Attempt was made to formulate a palm olein-based mould oil with an additive, benchmark its properties against the commercially used petroleum counterpart and evaluate its performance on-site intended for use in concrete manufacturing industry.

MATERIALS AND METHODS

Materials

Palm olein (iodine value = 56-58) was obtained locally from a palm oil refinery, after undergoing refining, bleaching and deodourising (RBD) processes. RBD palm olein was chosen on the basis of its lubrication effect and fluidity for ready formulation with additive unlike CPO, palm stearin (Afifah *et al.*, 2019) or waste cooking oil (Borugadda and Goud, 2016) which is deemed unsuitable before used due to physical appearance and quality issue. The specialty additive used, a National Sanitation Foundation (NSF)-certified ingredient for use in lubricants with incidental food contact (HX-1), *i.e.*, a food-grade tackifier (Functional-V584, Reg. No.

120913) (specific gravity = 0.93, flash point = 150°C, thickening efficiency = 55-70 cSt @ 40°C), was purchased commercially from Eweka International (Singapore) Pte. Ltd. The chosen NSF-certified tackifier at a permissible ± 10 vol.% is considered safe for workers in case of any incidental contact in and around the workplace. The petroleum-based mould oil in use by the collaborator in this study, a local manufacturer of concrete blocks as construction materials, has specifications as stipulated in *Table 1*. These specifications were referenced against the lubricating properties of the optimum blend of palm olein-based mould oil formulated in this study.

Methods

Viscosity and its relationship with temperature and pressure primarily affect the tribological performance of a lubricant (Chan *et al.*, 2018). In this study, palm olein was dosed with the tackifier, Functional-V584 to improve its viscosity desirable for mould oil formulation. No chemical/structural modification was required. Five different palm olein; tackifier ratios, *i.e.*, 95:5, 90:10, 85:15, 80:20 and 75:25 (v/v %) were used for optimisation study, in order to obtain a suitable blend with the targeted viscosity. Laboratory-scale optimisation was conducted by formulating five different blends of palm olein-based mould oil according to the above-mentioned palm olein: tackifier ratios using 250 mL beakers, followed by screening of the resulting kinematic viscosity of the blends. No software was used for the optimisation study. The ingredients were mixed and stirred vigorously at 100 rpm while keeping temperature between 50°C to 60°C for 30 min, and then stored in sealed amber glass bottles prior to kinematic viscosity analysis. The optimum ratio that gave as close to the kinematic viscosity of the petroleum counterpart as possible was screened and identified by withdrawing a small sample aliquot from each blend (20 mL) for the kinematic viscosity analysis. No further correlation was conducted on physical properties, friction and wear, *etc.*, for all the blends at this stage. Once the optimum blend was identified, large-scale mould oil production was conducted for field evaluation. A fabricated 200-L mild steel mixer tank (*Figure 1*) equipped with an agitator motor (0.4 kW/415 V), a heater and a programmable logic controller was designed and used to produce the intended mould oil based on the optimised palm olein: tackifier (90:10) blend. Both palm olein (180 L) and tackifier, Functional-V584 (20 L) were poured into the mixer tank. The tank was agitated and heated from room temperature to 60°C and maintained for 30 min to ensure complete mixing of the two ingredients. The homogenous solution was cooled to room temperature prior to storage for field trial. Palm olein: tackifier (100:0) and (95:5) blends were used as controls.

TABLE 1. LUBRICATING PROPERTIES[a] OF PALM OLEIN, PALM OLEIN-BASED MOULD OIL AND COMMERCIAL MOULD OIL

Property	Palm olein (pure blend)	Palm olein-based mould oil; palm olein: additive (90:10)[b]	Commercial mould oil
Density at 25ºC (kg L^{-1}), ASTM-D4052	0.8975	0.9090	0.8838
Viscosity, kinematic @ 40ºC/100ºC (cSt), ASTM D445	41.66/8.47	58.42/11.29	107.52/18.76
Viscosity index, ASTM D2270	186	191	195
Moisture content (%), ASTM D1744	0.056	0.5095	0.072
Saponifiable value (mg KOH g^{-1} oil), MPOB c2.9:2004	192	-	NA
Pour point (ºC), ASTM D97	6.0	6.0	-3.0
Cloud point (ºC), ASTM D2500	7.0	9.4	-5.2
Total acid number (mg g^{-1}), ASTM D664	4.60	0.80	3.92
Free fatty acids (%), MPOB c2.5:2004	0.079	0.760	NA
Copper strip corrosion, ASTM D130	1a	1a	1a
Oxidative stability (hr), EN 14112	25.30	22.83	NA
Oxidative stability by RPVOT (min), ASTM D2272	14	72	NA
Flash point (ºC), ASTM D93	305	340	246
Biodegradability @ 28 days incubation, OECD 301F	-	>60% (readily biodegradable)[c]	NA

Note: [a] Means reported, n=2; [b] Loh *et al.* (2010). The characteristics are typical of this batch production. Future product will conform to this specification, in which variations in the characteristics may occur; [c] Siti Afida *et al.* (2015); ASTM - ASTM International, formerly known as American Society for Testing and Materials; MPOB - Malaysian Palm Oil Board; KOH - potassium hydroxide; RPVOT - rotating pressure vessel oxidative test, OECD - Organisation for Economic Co-operation and Development; NA - not applicable.

Figure 1. A 200-L capacity mixer tank (a) front view, and (b) back view for blending of palm olein-based mould oil.

Analyses

The lubricating characteristics of palm olein and its optimum mould oil blend were measured according to standard test methods. Kinematic viscosity of the oil samples (20 g) at 40ºC and 100ºC was simultaneously measured by a dual-bath automated multi-range viscometer HVM472 (Walter Herzog, Germany) according to ASTM D445. Viscosity index was calculated based on ASTM D2270 from the resulted kinematic viscosities at 40°C and 100°C. Density was measured according to ASTM D4052 by a Mettler-Toledo digital density meter DE40 at 25ºC. Pour point and cloud point were measured by an automatic pour point/cloud point measuring apparatus (ISL CPP 97-2 Analyser) based on ASTM D97 and ASTM D2500, respectively while flash point measurement by a Pensky-Martens closed cup tester, ASTM D93 using an automated flash point tester (Petrotest, Germany). Moisture content was measured via the Karl Fisher method, ASTM D1744. Oxidative stability was determined via rotating pressure vessel oxidative test (RPVOT) using a quantum oxidation tester (Tannas, USA) according to ASTM D2272. Oil sample (50 g), water (5 mL) and a copper catalyst coil were placed in a stainless-steel pressure chamber which spun at a 30º angle, 100 rpm at 150ºC with O_2 supplied at 90 psi. The oxidation time of the test oil was determined when there was a drop of pressure of 25.4 psi in the chamber. Copper strip corrosion was performed on a Petrotest test bath (Germany) for 3 hr at 50°C, ASTM D130. The corrosion level was reported by comparing the tested copper strips with the standard. Total acid number was determined via potentiometric titration using Metrohm titrator based on ASTM D664. Free fatty acid (FFA) was measured using titration method according to MPOB Test Methods c2.5: 2004 (MPOB, 2005). Mixture of oil (20 g), neutralised *iso*-propanol (50 mL) and phenolphthalein indicator solution (2 mL) was stirred and heated on a hot plate at 40ºC in a 100-mL conical flask. Sodium hydroxide, 0.1 M, was titrated to the mixture, to a persistent permanent pink colour. The FFA content was calculated based on the percentage of palmitic acid. Saponifiable value (SV) was determined based on MPOB Test Methods c2.8: 2004 (MPOB, 2005). Mixture of oil (5 g) and ethanolic potassium hydroxide solution (50 mL) was boiled under reflux, then phenolphthalein indicator (1 mL) was added followed by titrating the excess potassium hydroxide with 0.5 M hydrochloric acid solution, to total disappearance of pink colour. The biodegradability of the optimum mould oil blend was tested for a 28-day incubation period based on Organisation for Economic Co-operation and Development (OECD) Test Guideline 301F Manometric Respirometry Test using the BOD EVO system (Velp, Italy). The procedure of the test and the method to calculate the biodegradability percentage can be referred to in Siti Afida *et al.* (2015). All determinations were performed in duplicate, and the mean values were reported. As a cost-savings measure, the lubricating properties of the rest of the formulated blends of palm olein-based mould oil in this study were not analysed except kinematic viscosity for optimum mould oil blend identification based on the guiding principle that it must exhibit the closet kinematic viscosity to that of the commercial counterpart.

In addition, oxidative stability via the Rancimat method was measured using a Model 743 Rancimat instrument (Metrohm AG, Switzerland). Oil sample (3 g) was analysed under a constant airflow of 10 L hr^{-1} at 110ºC heating temperature. The fatty acid compositions (FAC) of the samples were determined according to ISO 5508: Animal and vegetable fat

and oil analysis by gas-liquid chromatography. The measured FAC could be used to indicate and support the stability and cold flow property of the formulated mould oil. Analysis was carried out using a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionisation detector and a split injector. A fused silica capillary column (60 m × 0.25 mm) coated with a highly polar stationary phase, Supelco SP2340 (0.2 μm), was used with a programmed temperature profile as follows: oven temperature, 185°C; injector temperature, 240°C; detector temperature, 240°C; split ratio, 1:100; and carrier gas, helium at 2.0 mL min^{-1}. All determinations were performed in duplicate and only the means were reported.

Field Evaluation

The produced palm olein-based mould oil (100 L) was stored in drum and sent immediately to the collaborator's concrete manufacturing site for trials. The required conditions for commercial concrete production were: ~1.5 L mould oil per formwork; oven baking (moulding) temperature, 80°C and baking (drying) time, ~3.5 hr; autoclave curing temperature and time, 190°C and 7 hr, respectively. Ten consecutive formworks were operated in parallel during the trial. *Figure 2* illustrates the process flow during mould oil trial for concrete manufacturing at the production line. The mould oil was applied thoroughly to the inner walls of the formwork using a roller to form a thin oil film and any oil surplus was removed prior to pouring concrete mixer onto the formwork via a nozzle installed at the top until it was half filled. Since the concrete mixer was pushed down forcefully by gravity due to the height of the nozzle, mould oil with sufficient tackiness is required so that it will not be squeezed out leaving the formwork unlubricated. The concrete mixer slowly solidified upon completion of pouring. The moulded concrete was baked in an oven at 80°C ± 2°C, then cured in an autoclave at 190°C ± 5°C for 7 hr and cooled before the hardened concrete was detached from the formwork by turning the formwork upside down and unscrewed it. Visual observation was performed on the detached finished goods and the formwork. Field trial evaluation was performed on selected blends (palm olein: tackifier = 100:0, 95:5, 90:10 v/v%).

RESULTS AND DISCUSSION

In commercial concrete block production, a lubricant with desirable mould release property is particularly useful to facilitate process of detaching the moulded concrete from its formwork. The commonly used

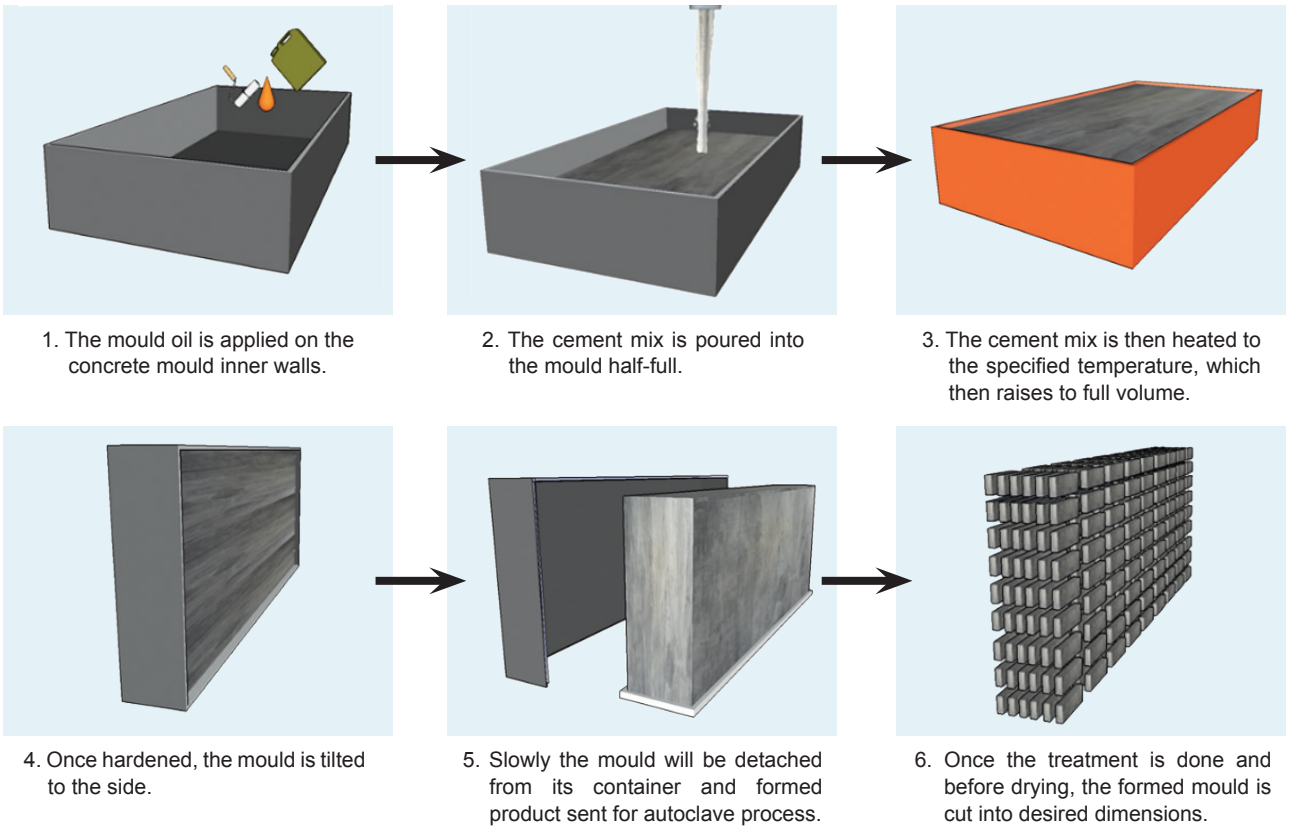


Figure 2. Concrete making lubricated by palm olein-based mould oil.

petroleum-based mould release agent (*Table 1*) exhibits chemical rather than a barrier (physical) release, lubricates and produces a smooth finish to the concrete, ensures less volume of lubricant used and protects mould casings from corrosion. It has high kinematic viscosity of 107.5 cSt at 40ºC and passes the copper strip corrosion test (ASTM D130). For the intended concrete blocks manufacturing, it is desirable to have a good mould release agent in fulfilling the three important characteristics, *i.e.*, lubricity, release ability and corrosion protection. Theoretically, in palm olein-based mould oil formulation, these important lubricating properties, in particular kinematic viscosity, must be met in order to yield a comparable product. Palm olein with a kinematic viscosity of 41.7 cSt at 40ºC can be enhanced using a tackifier. Although the kinematic viscosity of the formulated palm olein-based mould oil increased steadily (*Figure 3*) with increasing doses of tackifier, it was impractical and costly to use a high dose of 25 vol.% which could increase the kinematic viscosity to the desired 109 cSt at 40ºC comparable to the petroleum counterpart. As the attempt was to formulate an eco-friendly mould oil, the maximum dose could only be up to ±10 vol.% for any incidental contact during manufacturing. As such, 10 vol.% of tackifier was used in the palm olein-based mould oil formulation yielding just a half of the desired kinematic viscosity of 58.42 cSt without jeopardising its mould release performance as shown in the field trials conducted. The naturally inherited characteristics of palm olein as discussed in Loh and Choo (2012) and further elaborated in this study are capable of maintaining the lubricating properties required similar to the used petroleum counterpart, and for a reduced cost measure as well.

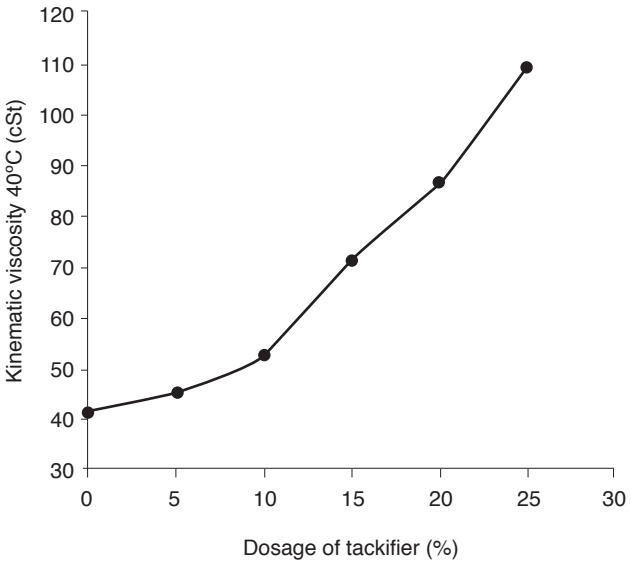
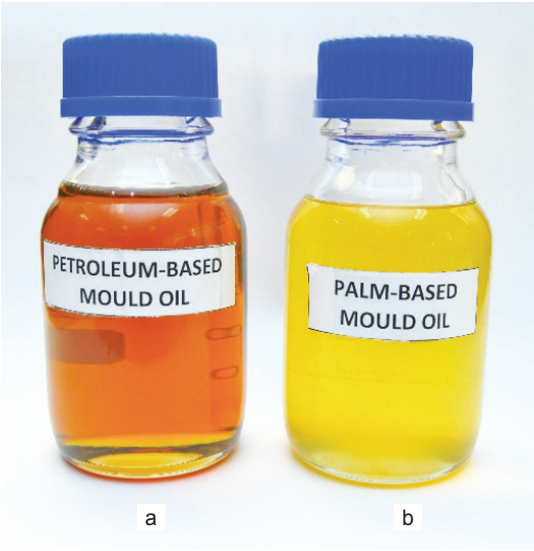


Figure 3. Influence of tackifier on kinematic viscosity of the palm olein-based mould oil (single measurement).

Characteristics of Palm Olein-based Mould Oil

The newly formulated palm olein-based mould oil (*Figure 4*) at an optimum 90:10 palm olein (as a base fluid): Tackifier blend was characterised. *Table 1* shows that the formulated mould oil had lubrication properties comparable to the petroleum-based commercial counterpart. Although palm olein was less viscous and could not meet the commercial specification of ~108 cSt, it could outperform in other aspects once a tackifier was added. In reality, additives are essential in almost any lubricants tailored to enhance key performance attributes. The formulated palm olein-based mould oil showed a moderate kinematic viscosity (58.42 cSt @ 40°C and 11.29 cSt @ 100°C) and a high viscosity index (191) which indicated good lubricity for the intended use. The oil was anticipated to experience very little viscosity changes with temperature effect owing to its high viscosity index. Its density was comparable to the benchmarked commercial mould oil but the moisture of 0.51% could imply that the oil might be prone to chemical decomposition known to affect its hydrolytic stability. The oil passed the copper strip corrosion test (1a), implying a non-corrosive substance used, as was evidenced by the low acid number of 0.8 mg KOH g^{-1} (*Table 1*).

The induction period (22.8 hr) via Rancimat test ensured that the formulation was stable and could withstand oxidation. The RPVOT time (14 min) of palm olein had been increased to 72 min after the formulation (*Table 1*). The RPVOT stability of the formulated palm olein-based mould oil was considered moderately well compared to those of soy-based chainsaw bar oil, 83 min (Cermak *et al.*, 2008); chemically modified castor-based



Source: Loh *et al.* (2010).

Figure 4. (a) Commercially used petroleum counterpart vs. *(b) palm olein-based mould oil formulated.*

lubricants, 43-150 min (Annisa and Widayat, 2018) and coconut oil-based estolites, 100-326 min (Cermak *et al.*, 2008), the latter two of which boosted with varying antioxidants. All vegetable oils without antioxidants have RPVOT times <20 min (Cermak *et al.*, 2008). The value is indicative of good degree of free-radicals (chemical) resistance, showing low oxidation tendency which reassures longer remaining useful life of the in-service oil. The oxidative stability of the above-mentioned plant oils, having either >90% unsaturation (soy and castor) or saturation (coconut) level, has been greatly improved via oligomerisation/esterification while the palm olein in this study can be used without chemical modification due to its appropriate saturated/unsaturated content to maintain a satisfactory oxidative stability. The major cause of poor oxidative stability of vegetable oil is FAC. According to Owuna *et al.* (2020), the fatty acids (unsaturation) present influences the thermal and oxidative stability of the formulated vegetable oil-based lubricant. Double bonds are susceptible to oxidation forming a series of corrosive oxygenated compounds. An oxidation-resistant lubricant will have fewer tendencies to form deposits, sludge and corrosive by-products during application. When oil is oxidised, it tends to thicken and polymerise and its functions degrade over time (Owuna *et al.*, 2020). In this study, the FAC profile (*Table 2*) of the formulated mould oil showed almost equal 45 saturated (C12, C14, C16, C18):54 unsaturated fatty acids (C18:1, C18:2, C18:3) level. This had rendered a fairly good oxidative stability of the mould oil, however, its cold flow properties, *i.e.*, pour point and cloud points might be compromised. It might not be suitable for use in cold environment and handling will be issue too. Formed wax crystals might clog filters and plug/openings leaving deposits on surfaces. Nevertheless, wax formation will not be encountered using palm oil in tropical climate (Liang *et al.*, 2016). The mobility and intermolecular interactions of oil molecules are restricted as temperature decreases due largely to crystalline molecular packing, which facilitates interlocking of the sharp needle-like triacylglycerol crystals. Although soybean, sunflower and rapeseed oils with >80% unsaturation (*Table 2*) show excellent cold flow properties (Quinchia *et al.*, 2012), they are more susceptible to oxidation. As palm olein exhibits iodine value of 57 (average) which falls within but at the lower end of the acceptable range (*i.e.*, 50 to 130) for biolubricants formulation, its ability to flow at low temperature is not as easy as in tropical climate. However, this range of iodine value has positively impacted the mould release capability during field trial where the moderate level of C=C double bonds present does not show much affinity in attracting metallic cationic molecules due to relatively lower level of π-bond reactivity. The ester linkages (*Figure 5*) provide a vulnerable site for microbial cells to initiate the biodegradation process of the ester-derived triacylglycerol fat molecules (Siti Afida *et al.*, 2015), which translates into very high biodegradability rates for ester-based biolubricants. Being renewable with high degree of biodegradability (*Table 1*), palm olein is therefore environmental-friendly and suitably used as a loss lubricant as any vapour generated and emitted during lubrication process will be non-toxic to the atmosphere.

Influence of Palm-based Mould Oil at Concrete-Formwork Interface

Palm olein with inherent lubricity property and corrosion inhibition capability (Hassan *et al.*, 2016; Loh and Choo, 2012), as shown in *Table 1*, is a potential alternative to petroleum-based or synthetic mould oil used in current concrete manufacturing workplace. It is found effective in preventing concrete adhesion to aluminum (Freedman, 1975) and other metals. As palm olein is naturally polar due to the bound fatty acids in the triglyceride backbone, it has strong adhesion

TABLE 2. COMPARISON OF FATTY ACID COMPOSITIONS (FAC) OF PALM OLEIN WITH OTHER VEGETABLE OIL

FAC (wt.%)	Palm olein (pure blend)[a]	Palm olein-based mould oil[a] Palm olein: additive (90:10)	Soybean oil	Rapeseed oil	Sunflower oil
				(Quinchia *et al.*, 2012)	
Lauric acid (C12:0)	0.3	-	-	-	-
Myristic acid (C14:0)	1.0	1.14	-	-	-
Palmitic acid (C16:0)	39.8	40.06	11.28	4.56	6.18
Stearic acid (C18:0)	4.4	3.62	2.70	-	2.16
Oleic acid (C18:1)	42.4	43.77	24.39	65.99	26.13
Linoleic acid (C18:2)	12.2	10.73	56.28	21.13	65.52
Linolenic acid (C18:3)	0.4	0.68	5.34	8.16	-
Arachidic acid (C20:0)	0.4	-	-	-	-

Note: [a] Means reported, n=2.

to and high affinity for metal surfaces, *i.e.*, the wall of the formwork (*Figure 5*). This could help in maintaining a thin film over the oil-formwork interface and therefore provides an improved lubricity (Gawrilow, 2003). With such inherent properties, palm olein presents more advantageous as a mould release lubricant than its petroleum counterparts.

Besides, palm olein with SV of 186-198 mg KOH g^{-1} oil is desirable to perform mould release characteristic (Gawrilow, 2003). SV indicates the degree of a lipid in undergoing hydrolysis under basic condition to form a soap. Owing to naturally occurring triglyceride-based fatty acids, this range of SV tends to facilitate metal soap formation between oil (esters) molecules and the alkaline concrete aggregates (containing metal ions). In general, acceptable SV of plant oils is ranged 176-244 mg KOH g^{-1} oil (Gnanasekaran and Chavidi, 2018; Joseph and Saxena, 2017). A higher SV range is undesirable as it mobilises the concrete particles within the oil-formwork interface, thus softening takes place resulted in concrete sticking on formwork and striping is difficult (Bouharoun *et al.*, 2012; Freedman, 1975). The saponification ability of a vegetable esters in forming soap layers at the formwork interface is an added advantage for surface release lubrication, as oil participates in the formation of interstitial mediums creating a stable emulsion in the vicinity of formwork (Bouharoun, 2016).

In a similar manner, as the palm olein-based mould release agent formulated has naturally inherited fatty acids as a foreign (added) acidifier, as opposed to the petroleum counterparts, it shows a reduction in friction, *i.e.*, better release of mould and a high resistance to charge transfer. The presence of incoming fatty acids (surface active additive) is anticipated to catalyse greater soap formation (with basic charged metal ions), leading to a specific interface orientation of esters and fatty acid molecules in layers. The existence of different forms and strength of functional linkages due to different degrees of polarity between the oil and fatty acids has resulted in higher tendency of the latter to compete and orient preferentially as soap film in the concrete-oil-formwork interface (*Figure 6*), where chemical effect has taken precedence over the physical effect. As a result, it reduces and relaxes the interfacial tension (Djelal *et al.*, 2008), and hence enabling mould release ability and performance. This characteristic has greatly succeeded in overcoming major shortcoming of palm olein-based mould oil deployment considering its borderline kinematic viscosity for the intended commercial application.

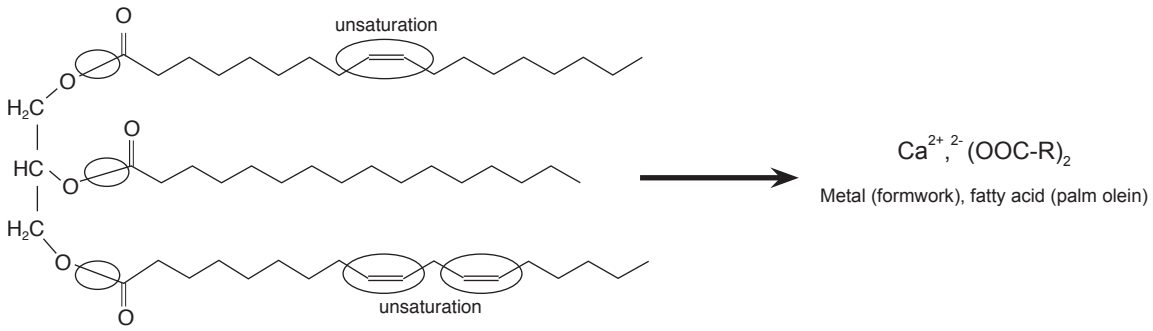


Figure 5. Interaction of a fatty acid or ester bound vegetable oil (left) with the metal surface of a formwork in performing a release property.

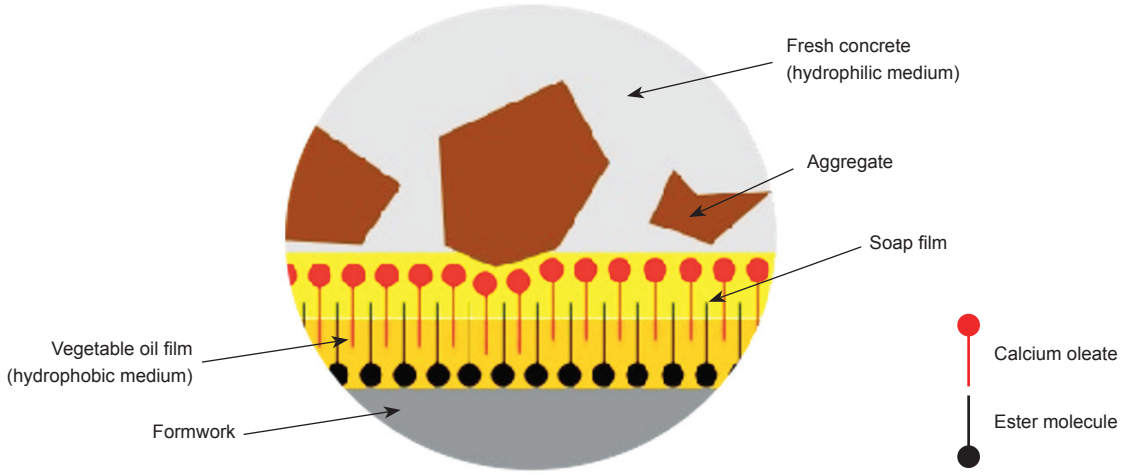


Figure 6. Organisation of a soap film at the concrete-oil-formwork interface (adopted from Bouharoun et al.*, 2012).*

Performance Evaluation

The performance of a mould oil is indicated by the condition of the moulded concrete after being detached from its formwork. The method of applying mould oil on the wall formwork is particularly important as evenly and thinly distributed oil conditions the quality of the concrete facing. The mould oil, when applying evenly to a clean surface, creates a continuous and uniform film. On the contrary, untouched wall will form bonding to concrete products being made.

Besides having a minimal viscosity range desirable for concrete working conditions, the palm olein-based mould oil is capable of remaining in place to perform the required lubricity and mould release property within the concrete-oil-formwork interfaces. As the naturally-occurring and polarised ester linkage in palm oil imparts good lubricity (Gawrilow, 2003; Loh and Choo, 2012), it can withstand friction stress or pushing forces (Hassan *et al.*, 2016) incurred during pouring of the concrete onto the formwork. Hence, it provides and maintains a thin and continuous film over the oil-formwork interface, to smoothen, lubricate and release the moulded concrete easily. An overdosing or excess oil on the formwork causes poor concrete facing quality as it gives rise to softening and uncooked concrete blocks with obvious oil stain. The deriving low acid number of 0.8 mg KOH g^{-1} (*Table 1*) implies less corrosive properties (Owuna *et al.*, 2020) and hydrolysis of esters in water, if any, while performing the desired mould release.

The field trials of the selective mould oil blends at palm olein: tackifier 100:0, 95:5, 90:10 (v/v%) were evaluated on-site at a concrete production factory. The resulted concrete blocks using 100% palm olein blend (negative control) showed inferior quality with noticeable soft concrete surfaces that retained the mould oil as oil dots on the surfaces of the blocks (bottom and both ends of the block) (*Figure 7a*). The oil dot area was obviously not cooked and soft with a depth of approximately 3-5 mm. As a pure blend, it was less viscous (42 cSt) compared to palm olein-based (58 cSt) and petroleum-based mould oil (108 cSt), hence some of the concrete surfaces were not fully covered and lubricated, causing surfaces deterioration or damage and softness. Accepting the fact that almost all formulated lubricant systems in the market require additives to be on par for high performance compatibility, a tackifier is undoubtedly needed to increase the viscosity inadequately performed by palm olein which subsequently enhance the mould release property.

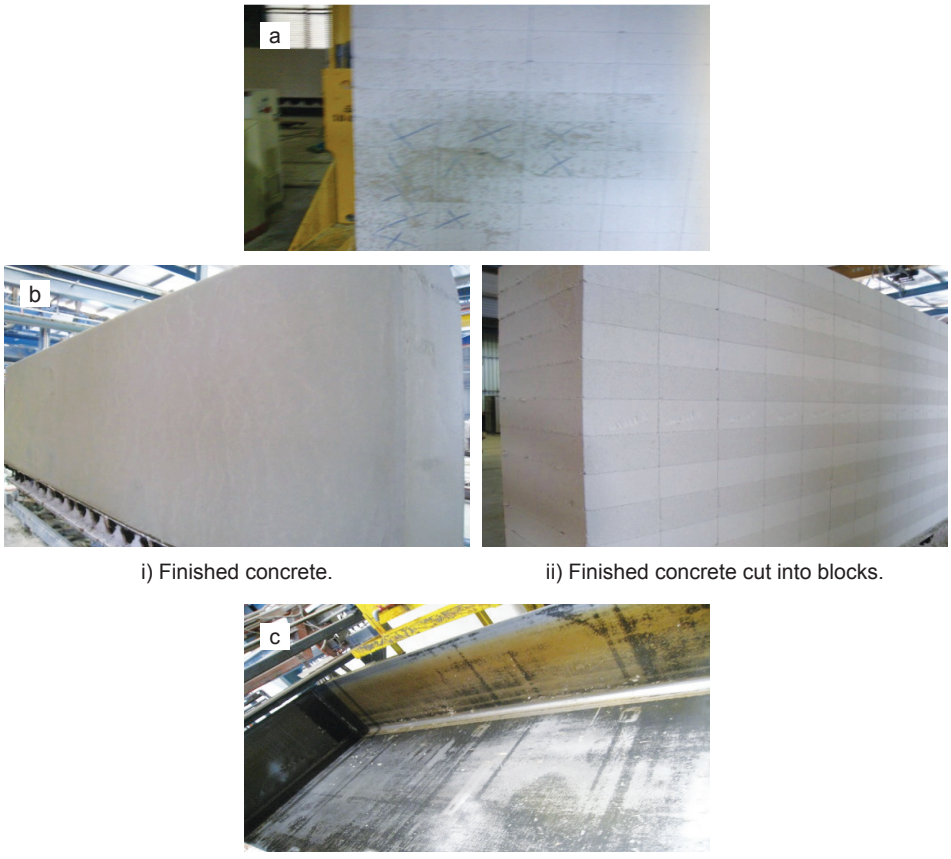


Figure 7. Mould oil performance on finished concrete blocks (a) oil stain (visibly darker), (b) clean surface and (c) no concrete leftover sticking in the inner wall or surface of the mould car.

However, by adding 5 vol.% of the tackifier into palm olein, the resulted finished products failed to show any improvement. Lastly, the prior identified 90:10 palm olein: tackifier optimum blend (based on kinematic viscosity) showed very promising results. The finished concrete blocks showed no oil stain on concrete surfaces (*Figure 7b*) and no breakage (broken edges or broken corners) during cake demoulding; and the formwork showed smooth surfaces without cement sticking onto the sides and bottom (*Figure 7c*). As mentioned earlier, relatively, though ester-based biolubricants might be more susceptible to hydrolytic attack and prone to issue on material-lubricant compatibility (Ho *et al*., 2019), the performance of the respective palm olein-derived mould release agent was not affected even in the presence of water in the concrete mixer.

The potential of palm olein as a base stock for mould oil formulation was evident from the findings of the field trials conducted in this study as concluded based on visual observation and assessment. In this study, temperature range for concrete moulding process was 80°C (baking) and 190°C (curing) to shape and harden the formed concrete. The formulated mould oil has a flash point, 340°C, much higher than the moulding temperature, so there will be no flammability issue as the temperature experienced will not be able to vapourise the oil to form an ignitable mixture. The resulting low volatility will not pose fire hazard at workplace and can prolong its use as a total loss lubricant. The tribological and rheological behaviour and many other physical aspects relating to facing quality and appearance plus storage performance as demonstrated by Libessart *et al.* (2014) should be investigated and substantiated in the near future to better understand the type of new lubricant product family involved for market entry and acceptance.

For cost-benefit concrete production, the production line of mould oil should be located adjacent to concrete blocks manufacturing lines to allow for immediate and direct application of freshly made mould oil. Besides being environmental-friendly and biodegradable, the formulated palm olein-based mould oil could offer less hazard, hassle and maintenance in the workplace, do away with irritating odour and flammable gas, provide compatible formed products quality, enhance the green image of the industry, *etc.* More importantly, the mould oil formulated is an eco-friendly biodegradable lubricant able to meet the HACCP system requirement widely enforced in today's good manufacturing industrial practices. Commercialisation efforts in recent years have shown that the palm olein-based mould oil is 20%-30% lower compared to market price. The findings are expected to demonstrate and guide the agricultural sector (*e.g.*, oil palm industry players and farmers) in adopting resource diversification measure so as to utilise resource optimally and create value addition in order to support sustainable agriculture and a circular economy.

CONCLUSION

An eco-friendly biodegradable palm olein-based mould oil was formulated having >60% ready biodegradability in aquatic environment within 23 days test period. Besides offering a decent workplace to workers, it performed satisfactorily as a mould release lubricant fulfilling every aspect of the specifications for mould oil used in concrete production sites besides ensuring that the production process and the finished concrete products conforming to higher standards and more stringent conditions and regulations. Both the finished concrete blocks and formwork showed smooth finishing without any underlying defect when applied with the 90:10 palm olein; tackifier optimum blend. Besides non-toxic in nature, its simple, straightforward and economical manufacturing process has driven it more appealing than the commercially available petroleum-based counterparts, as an emerging green product for not just the palm oil industry players but also other manufacturing industries gearing towards meeting the HACCP requirement. Future research should focus on quality of concrete surfaces to better understand the effects of concrete/oil/formwork interface phenomena.

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REFERENCES

Afifah, A N; Syahrullail, S; Azlee, N I W; Sidik, N A C; Yahya, W J and Rahim, E A (2019). Biolubricant production from palm stearin through enzymatic transesterification method. *Biochem. Eng. J., 148*: 178-184.

Annisa, A N and Widayat, W (2018). A review of bio-lubricant production from vegetable oils using esterification transesterification process. *MATEC Web of Conferences 156*: 06007. DOI: 10.1051/matecconf/201815606007.

Argus Media (2021). Malaysia's Covid lockdown squeezes UCO supplies. https://view.argusmedia.com/APAC-EMD-2021-01-BIO-

Argus-Biofuels_01-News-Story-1.html, assessed on 18 May 2021.

Borugadda, V B and Goud, V V (2016). Improved thermo-oxidative stability of structurally modified waste cooking oil methyl esters for bio-lubricant application. *J. Clean. Prod., 112(5)*: 4515-4524.

Bouharoun, S (2016). Characterization of the interface between fresh concrete and formwork. *J. Civ. Eng. Manag., 22(1)*: 26-37. DOI: 10.3846/13923730.2014.893912.

Bouharoun, S; Vanhove, Y; Djelal, C; De Caro, P and Dubois, I (2012). Interactions between superplasticizer and release agents at the concrete/formwork interface. *Mater. Sci. Appl., 3*: 384-389.

Cermak, S C; Biresaw, G and Isbell, T A (2008). Comparison of a new estolide oxidative stability package. *J. Am. Oil Chem. Soc., 85(9)*: 879-885. DOI: 10.1007/s11746-008-1265-3.

Chan, C H; Tang, S W; Mohd, N K; Lim, W H; Yeong, S K and Idris, Z (2018). Tribological behavior of biolubricant base stocks and additives. *Renew. Sustain. Energ. Rev., 93*: 145-157.

Dandan, A and Samion, S (2017). Palm oil as an alternative lubricant oil: A brief review. *The Colloquium, 11*: 17-19.

Djelal, C; De Caro, P; Libessart, L; Dubois, I and Pebere, N (2008). Comprehension of demoulding mechanisms at the formwork/oil/concrete interface. *Mater. Struct., 41*: 571-581.

EuropaLub (2015). EuropaLub Issue 1. Launch presentation. 2015. European Lubricants Statistical Service.

Farfan-Cabrera, L I; Gallardo-Hernández, E A; Gómez-Guarneros, M; Pérez-González, J and Godínez-Salcedo, J G (2020). Alteration of lubricity of *Jatropha* oil used as bio-lubricant for engines due to thermal ageing. *Renew. Energ., 149*: 1197-1204.

Fernandes, K V; Cavalcanti, E D C; Cipolatti, E P; Aguieiras, E C G; Pinto, M C C; Tavares, F A; Silva, P R da; Fernández-Lafuente, R; Arana-Peña, S; Pinto, J C; Assunção, C L B; Silva, J A C da and Freire, D M G (2021). Enzymatic synthesis of biolubricants from by-product of soybean oil processing catalyzed by different biocatalysts of *Candida rugosa* lipase. *Catal. Today, 362*: 122-129.

Fernandes, K V; Papadaki, A; Silva, J A C da; Fernandez-Lafuente, R; Koutinas, A A and Freire, D M G (2018). Enzymatic esterification of palm fatty-acid distillate for the production of polyol esters with biolubricant properties. *Ind. Crop. Prod., 116*: 90-96.

Freedman, S (1975). Part I of a series on form release agents and form coatings, Publication #C750084, The Aberdeen Group.

Gawrilow, I (2003). Palm oil usage in lubricants. Paper presented at the 3rd Global Oils and Fats Business Forum USA. Interfacing with the Global Oils and Fats Business. 8 October 2003.

Gnanasekaran, D and Chavidi, V P (2018). Vegetable oil-based bio-lubricants and transformer fluids. *Book Series: Materials Forming, Machining and Tribology*. Springer Nature, Singapore. 155 pp.

Grand View Research (2016). Biolubricants market size to reach $2.92 billion by 2024. https://www.grandviewresearch.com/press-release/bio-lubricants-market, accessed on 1 September 2016.

Hassan, M; Ani, F N and Syahrullail, S (2016). Tribological performance of refined, bleached and deodorised palm olein blends bio-lubricants. *J. Oil Palm Res., 28(4)*: 510-519.

Ho, C K; McAuley, K B and Peppley, B A (2019). Biolubricants through renewable hydrocarbons: A perspective for new opportunities. *Renew. Sust. Energ. Rev., 113*: 109261.

Hussein, R Z K; Attia, N K; Fouad, M K and El Sheltawy, S T (2021). Experimental investigation and process simulation of biolubricant production from waste cooking oil. *Biomass Bioenerg., 144*: 105850.

Joseph, P V and Saxena, D (2017). Thermo-oxidatively improved environmental friendly base oils. STLE Atlanta. 21-25 May 2017.

KKM (2018). *Kementerian Kesihatan Malaysia, Bahagian Keselamatan dan Kualiti Makanan*, MeSTI. http://fsq.moh.gov.my/v6/xs/page.php?id=208 and http://fsq.moh.gov.my/v6/xs/page.php?id=203, accessed on 17 May 2021.

Kheang, L S; May, C Y and Ngan, M A (2007). Residual oil from spent bleaching earth (SBE) for biodiesel and biolubricant applications. *MPOB Information Series No. 381*.

Lafay, V S and Neltner, S L (2002). A biodegradable release agent increasing the life of concrete forms. US Patent: 2002172759 A1.

Liang, Y C; Loh, S K; Soon, L W and May, C Y (2016). Malaysian B5 implementation and its quality. *J. Oil Palm Res., 28(3)*: 331-343.

Libessart, L; Djelal, C and de Caro, P (2014). Influence of the type of release oil on steel formwork corrosion and facing aesthetics. *Constr. Build. Mater., 68*: 391-401.

Lightcap, D V (1997). Vegetable oil-based sealing compositions for concrete. US Patent: 5647899 A.

Loh, S K; Chew, S M and Choo, Y M (2006). Oxidative stability and storage behavior of fatty acid methyl esters (FAME) derived from used palm oil. *J. Amer. Oil Chem. Soc., 83*: 947-952.

Loh, S K and Choo, Y M (2012). Influence of a lubricant auxiliary from palm oil methyl esters on the performance of palm olein-based fluid. *J. Oil Palm Res., 24*: 1388-1396.

Loh, S K; Muzzammil, N and Choo, Y M (2010). Palm-based mould oil for the steel/concrete industry. *MPOB Information Series No. 521*.

MPOB (2005). A compendium of test on palm oil products, palm kernel products, fatty acids, food related products and others. *MPOB Test Methods* (Aini, K; Siew, W L; Tan, Y A; Nor Aini, I; Tang, T S and Nuzul, A I eds.). MPOB, Bangi.

Mobarak, H M; Niza Mohamad, E; Masjuki, H H; Kalam, M A; Al Mahmud, K A H; Habibullah, M and Ashraful, A M (2014). The prospects of biolubricants as alternatives in automotive applications. *Renew. Sust. Energ. Rev., 33*: 34-43.

Nielsen, E (1985). Composition for release of a molded concrete body from the mold. PCT Int. Application: WO 8505066 A1.

Owuna, F J; Dabai, M U; Sokoto, M A; Dangoggo, S M; Bagudo, B U; Birnin-Yauri, U A; Hassan, L G; Sada, I; Abubakar, A L and Jibrin, M S (2020). Chemical modification of vegetable oils for the production of biolubricants using trimethylolpropane: A review. *Egypt. J. Pet., 29(1)*: 75-82.

Pindit, K; Thanapimmetha, A; Saisriyoot, M and Srinopakun, P (2021). Biolubricant basestocks synthesis using 5-step reaction from jatropha oil, soybean oil, and palm fatty acid distillate. *Ind. Crop. Prod., 166*: 113484.

Pirow, R; Blume, A; Hellwig, N; Herzler, M; Huhse, B; Hutzler, C; Pfaff, K; Thierse, H-J; Tralau, T; Vieth, B and Luch, A (2019). Mineral oil in food, cosmetic products, and in products regulated by other legislations. *Crit. Rev. Toxicol., 49(9)*: 742-789. DOI: 10.1080/10408444.2019.1694862.

Quinchia, L A; Delgado, M A; Franco, J M; Spikes, H A and Gallegos, C (2012). Low-temperature flow behaviour of vegetable oil-based lubricants. *Ind. Crop. Prod., 37(1)*: 383-388.

Rac, A and Vencl, A (2008). Performance investigation of chain saw lubricants based on new sunflower oil (NSO). *Proc. of the OTG-Symposium 2008*. Wr. Neustadt, Austria. p. 249-257.

Rao, T V V L N; Rani, A M A; Awang, M; Baharom, M and Uemura, Y (2018). An overview of research on biolubricants in Malaysia and Japan for tribological applications. *Jurnal Tribologi, 18*: 40-57.

Research and Markets (2019). 2019 Report: Global lubricants market size is projected to reach USD188.0 billion by 2024 from USD164.3 billion in 2019, at a CAGR of 2.7%. https://www.prnewswire.com/news-releases/2019-report-global-lubricants-market-size-is-projected-to-reach-usd-188-0-billion-by-2024-from-usd-164-3-billion-in-2019--at-a-cagr-of-2-7-300951823.html, accessed on 18 May 2021.

Sahara Business Group (2021). Global lubricant demand. https://saharabizz.com/1279-2/, accessed on 18 May 2021.

Shah, R; Woydt, M and Aragon, N (2020). Where are lubricants headed? *Inform, 31(10)*: 12-15.

Singh, R; Lee, K T; Ooi, L C L; Low, E T L; Abdullah, M O; Sambanthamurthi, R and Azman, I (2021). An overview of the development of the oil palm industry and impact of the shell gene innovation as a quality control tool to improve productivity. *J. Oil Palm Res*. DOI: 10.21894/jopr.2021.0001.

Siti Afida, I; Razmah, G; Yeong, S K and Hazimah, A H (2015). Biodegradability of palm-based lubricants. *J. Oil Palm Res., 27(4)*: 425-432.

Sönnichsen, N (2021). Global lubricant demand 2000-2019. https://www.statista.com/statistics/411616/lubricants-demand-worldwide/#statisticContainer, accessed on 18 May 2021.

Souza, M C de; Gonçalves, J F de S; Gonçalves, P C; Lutif, S Y S and Gomes, J de O (2019). Use of *Jatropha*

and *Moringa* oils for lubricants: Metalworking fluids more environmental-friendly. *Ind. Crop. Prod., 129*: 594-603.

Srivastava, A and Sahai, P (2013). Vegetable oils as lube basestocks: A review. *Afr. J. Biotechnol., 12(9)*: 880-891.

Sukirno and Farhandika, L (2020). Synthesis and characterisation of ethylene glycol ester from spent bleaching earth oil and ethylene glycol as hydraulic lubricants. *AIP Conf. Proc., 2255*: 060015. DOI: 10.1063/5.0021783.

Syahrullail, S; Zubil, B M; Azwadi, C S N and Ridzuan, M J M (2011). Experimental evaluation of palm oil as lubricant in cold forward extrusion process. *Int. J. Mech. Sci., 53(7)*: 549-555.

Theodori, D; Saft, R J; Krop, H and Van Broekhuizen, P (2004). Background document on development of criteria for the award of the European Eco-label to lubricants. https://ec.europa.eu/environment/archives/ecolabel/pdf/lubricants/2ndbackdoc_1st criteria_def.pdf, accessed on 18 May 2021.

Wittich, L; Heck, S; Friesenhagen, L; Demmering, G; KomP, H-D and Koehler, M (1995). Emulsions for separating concrete forms. German Patent: 4400272 A1.

Woittiez, L S; Wijk, M T; Slingerland, M; Noordwijk, M and Giller, K E (2017). Yield gaps in oil palm: A quantitative review of contributing factors. *Eur. J. Agron., 83*: 57-77.

Woma, T Y; Lawal, S A; Abdulrahman, A S; Olutoye, M A and Ojapah, M M (2019). Vegetable oil based lubricants: Challenges and prospects. *Tribol. Online, 14(2)*: 60-70.

Yi, R C (1996). A preparation of mold-releasing agent for concrete from industrial wastes. China Patent: 1129633.

Zhang, W; Ji, H; Song, Y; Ma, S; Xiong, W; Chen, C; Chen, B and Zhang, X (2020). Green preparation of branched biolubricant by chemically modifying waste cooking oil with lipase and ionic liquid. *J. Clean. Prod., 274*: 122918.

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INFLUENCE OF CHEMICAL COMPOSITION OF ACTIVATED CALCIUM BENTONITES AND SODIUM BENTONITES ON PALM OIL BLEACHING CAPACITY AND OIL QUALITY

DOLPHEN, R[1]; DHURAKIT, P[2] and THIRAVETYAN, P[2]*

ABSTRACT

The efficiency of clays in bleaching degummed palm oil depends on their unique characteristics. This study sheds new light on a novel characteristic which impacts on the quality and bleaching capacity of activated clay. Although bentonites may originate from different areas, their structural modifications can make them ideally suited for bleaching. Calcium (Ca)-bentonites and sodium (Na)-bentonites, including activated forms of both clays, were investigated in depth. Interestingly, X-ray fluorescence (XRF) spectrometry indicated that the high bleaching capacity of Na-bentonite was correlated with silica (SiO_2) and alumina (Al_2O_3) contents in the range of 68.90%-85.20% and 8.96%-16.60% by weight (wt), respectively. The results showed that Na-bentonite treated with 1.5 M sulphuric acid (H_2SO_4) at a clay:acid ratio of 10:50 (w v^{-1}) and refluxing time of 8 hr had a higher bleaching capacity (78.04%) than commercial clay (67.09%). These characteristics can provide a suitable reaction space at the interlayer for adsorption of pigments and impurities. Moreover, the specific surface area and total pore volume of this activated clay also increased. After bleaching by treated Na-bentonite with 1.5 M H_2SO_4, degummed palm oil appeared to be of good quality, leading to less deterioration and rancidity due to decreased free fatty acid (FFA), unsaturated fatty acid, iron (Fe) and phosphorus (P) contents.

Keywords: bleaching, calcium bentonite, colour, palm oil, sodium bentonite.

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INTRODUCTION

Crude palm oil (CPO) is found to contain pigments, such as carotenoids, especially β-carotene, and their derivatives xanthophylls, chlorophyll, pheophytin, tocopherols and gossypol, as well as oxidised fatty acids, trace soaps and trace metals [copper ions (Cu^{2+}), ferric ions (Fe^{3+})] (Rossi *et al.*, 2011), which negatively influence the taste, smell and colour of the oil. Thus, removal of these substances causes a light yellowish colour and improves the stability and sensory quality of the oil for greater acceptance by consumers.

Bleaching is one of the most important steps in vegetable oil refining; in this step, pigments and undesirable impurities are removed by a process involving van der Waals forces and covalent bonds (Nwabanne and Ekwu, 2013). The efficiency of palm oil bleaching has been reported in many types of adsorbents such as palm oil boiler ashes, activated coconut pod ash, perlite, smectite, activated kaolinite, pyrolysis waste material peanut hulls, press mud, rice husks and synthetic

[1] Pilot Plant Development and Training Institute, King Mongkut's University of Technology Thonburi, Bangkok 10150, Thailand.

[2] Division of Biotechnology, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkok 10150, Thailand.

* *Corresponding author e-mail: paitip.thi@kmutt.ac.th*

silica) (Ismail *et al.*, 2016; Lau *et al.*, 2019; Meesuk and Seammai, 2010; Rossi *et al.*, 2003; Tebandeke *et al.*, 2014; Warasith and Goodman, 2020). However, adsorption materials used nowadays throughout the world by vegetable oil refiners are mainly activated montmorillonite clays due to its structural unit consisting of an octahedral alumina sheet sandwiched between two sheets of tetrahedral silica. Bentonite consists mostly of montmorillonite and has been widely employed in refining edible oil because its swelling, adsorption capacity and surface acidity properties make it suitable for wide variety of applications (Ayari *et al.*, 2005). Numerous literature reports affirm that the activation of bentonites with acids such as sulphuric, hydrochloric and oxalic acids, as well as anionic and cationic surfactants, could increase their adsorbent properties (Gunawan *et al.*, 2010; Joy *et al.*, 2007; Salawudeen *et al.*, 2007; Warasith and Goodman, 2020). Activation is expected to improve certain features of clay, such as its specific surface area, porosity, functional groups and framework collapse and, thereby, its bleaching capacity (Aung *et al.*, 2014; Joy *et al.*, 2007; Kashani Motlagh *et al.*, 2011; Nde *et al.*, 2019; Nwabanne and Ekwu; 2013; Silva *et al.*, 2013; Usman *et al.*, 2013). As mentioned above, various research efforts on the best conditions for modified clays and then new bleaching clays were investigated in terms of their characteristics and mechanisms for bleaching oil. However, it is difficult to control the quality of clays after modification because they originate from various locales, which affects their properties (Afolabi *et al.*, 2017). Therefore, this study sheds new light on a novel characteristic of bentonites which impacts the quality and bleaching capacity of activated clay. Although bentonite clays are sourced from different areas, the preparation of clays should result in suitable amounts of the major constituents, especially silica (SiO_2) and alumina (Al_2O_3). There are no reports about the influence of the chemical composition of bentonites after activation on bleaching capacity. For instance, in the case of kaolin, which has a different clay structure from that of bentonite, suitable SiO_2 and Al_2O_3 contents of activated kaolin for rice bran oil bleaching were approximately 62.40%-65.10% and 31.20%-4.20%, respectively (Aung *et al.*, 2015).

Therefore, the aim of this research was to determine a suitable chemical composition in the structure of activated Ca-bentonites and Na-bentonites to predict clay quality in the bleaching process. The correlation between unique characteristics such as chemical composition (SiO_2, Al_2O_3, iron (III) oxide (Fe_2O_3) and magnesium oxide (MgO) contents) in clays and bleaching capacities were evaluated. Meanwhile, the crystal structure, surface area and pore volume were characterised to support the properties of the clays. In addition, aspects of oil quality after bleaching, such as free fatty acids (FFA), moisture content, peroxide value, iodine value and iron (Fe) and phosphorus (P) contents, were investigated.

MATERIALS AND METHODS

Materials

Samples of Ca-bentonite and Na-bentonite were obtained from Thep Agricultural Industry Co., Ltd., Thailand. The clay samples were dried at 70ºC for 24 hr and sieved through 200 mesh to obtain particles (75 μm). Commercial bleaching clay (montmorillonite) obtained from Taiko Clay Marketing, Malaysia was used as the reference clay for the bleaching tests. Degummed palm oil was obtained from Oleen Co., Ltd., Thailand. This oil had an orange-red appearance and contained 49.9 red and 5.9 yellow Lovibond units (Lovibond Tintometer Model F, Tintometer Ltd., United Kingdom).

Refluxing Process

Ten-gram bentonite samples were introduced into a round-bottom flask, 500 mL of sulphuric acid (H_2SO_4) in concentrations of 0.2-5.0 M was added to give a 1:50 (w v^{-1}) clay:acid ratio, and the mixture was then heated at 90°C for 4 hr under agitation. In the case of pre-heated materials, bentonites were prepared by heating in a muffle furnace at 300°C-700°C for 1 hr. Then, 10 g of each heated sample was refluxed with 500 mL of 2.0 M H_2SO_4 in a round-bottom flask under agitation at 90°C for 4 hr. Sodium bentonite samples were treated with various H_2SO_4 concentrations (0.6-3.0 M) at clay:acid ratios of 1:50, 5:50 and 10:50 (w v^{-1}), followed by refluxing at 90°C under agitation. The refluxing time was varied between 4, 6, 8 and 10 hr.

Next, the samples were washed several times with distilled water until the solution reached pH 3, corresponding to the optimum pH for bleaching of vegetable oil (Girgis, 2005). Subsequently, the samples were oven-dried at 80ºC for 24 hr to reduce the moisture content, crushed into a powder form and sieved through 400 mesh. All samples were stored at room temperature for further experiments.

Bleaching Experiments

One-hundred-milligram clay samples were added into 10 mL of CPO, and then the mixtures were heated at 90ºC in an oil bath for 30 min under constant agitation. After that, the oil samples were centrifuged at 4500 rpm for 15 min and then filtered through Whatman No. 5 filter paper. The bleaching process was performed in triplicate. Finally, the

colours of the palm oil samples were measured in a spectrophotometer at 450 nm (Thermo Scientific™, type Evolution 201, Waltham, MA, USA). The bleaching capacities were calculated according to Equation (1):

$$\text{Bleaching capacity } (\%) = [(A_0 - A)/A_0] \times 100 \quad (1)$$

where A_0 and A are the absorbances of the unbleached and bleached oil at 450 nm, respectively.

Characterisation of Clays by X-ray Diffraction (XRD), X-ray Fluorescence (XRF) and Measurement of Pore Structure

The XRD patterns of samples were recorded using a D8 Advance powder diffractometer (Bruker AXS, Germany) with Cu-Kα radiation at $\lambda = 1.540\,56$ Å, operated at 40 kV and 40 mA in the range of 10°-100°. A wavelength-dispersive X-ray fluorescence (WD-XRF) spectrometer (model S4 Pioneer, Bruker AXS, Germany) was used to determine the elements present in clay samples. The XRF spectrometer was equipped with a 4-W Rh anode X-ray tube, 60-kV generators, and eight diffracting crystals of various diffraction spacings. The porous properties of clay samples were measured by the adsorption-desorption isotherms of nitrogen gas with a surface area analyser (Autosorb-1, Quantachrome Instruments, USA). The Brunauer-Emmett-Teller (BET) equation was applied to estimate the specific surface area, total pore volume, micropore volume and average pore size of samples.

Physicochemical Characterisation of Palm Oil

Initial crude oils and bleached oils were characterised by assessment of moisture content (method Ca 2c-25), FFA (method Ca 5a-40), iodine value (method Cd 1c-85), peroxide value (method 965.32) and fatty acid composition (method Ce 1e-91) (Cunniff, 1997; Firestone, 1997). The colour of oil samples was estimated using a 5 ¼" path length and a Lovibond Tintometer Model F, according to method Cc 13b-45 (Firestone, 1997). In addition, the oil samples were ashed according to the AOAC 999.11 method for Fe and P analysis (Jorhem, 2000). Fe content was determined using inductively coupled plasma optical emission spectrometry (ICP OES; Horiba, JY2000, Japan), while the P content was determined by the colourimetric method.

Statistical Analysis

The data were statistically analysed by one-way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS). Significantly, different means were assessed by Duncan's multiple range test ($p<0.05$).

RESULTS AND DISCUSSION

Bentonite is aluminium phyllosilicate clay that consists mostly of montmorillonite but also contains impurities and is classified into Na or Ca types, depending on the dominant exchangeable cations (Hassan and Abdel-Khalek, 1998). Based on this classification procedure, Ca-bentonite and Na-bentonite were categorised as calcium (Ca-bentonite) and sodium Na-bentonite) types, respectively. In this study, however, the XRD pattern indicated that montmorillonite was a major component of bentonites (*Figure 1*).

Commercial clay (montmorillonite) has a bleaching potential for degummed palm oil of approximately 67.09%, while original of Ca-bentonite and Na-bentonite can bleach this oil approximately 9.02% and 0.89%, respectively. This may be due to the unique characteristics of these clays. Commercial clay, Ca-bentonite, and Na-bentonite are mainly composed of SiO_2, Al_2O_3 and Fe_2O_3. The SiO_2 and Al_2O_3 contents in commercial clay used for bleaching degummed palm oil are 80.70% and 10.20%, respectively. The XRF results of commercial clay showed a higher SiO_2 content (80.70%) than was found in Ca-bentonite (66.40%) and Na-bentonite (48.40%), while Al_2O_3 (10.20%) showed a lower content than was found in Ca-bentonite and Na-bentonite (20.30% and 19.40%, respectively) (*Figures 2* and *3*).

The element composition and content of clay structure can change the clay's unique characteristics. Interestingly, this study proposes that the bleaching capacity of degummed palm oil is related to suitable SiO_2 and Al_2O_3 contents. After acid activation, the crystallinity of Ca-bentonite and Na-bentonite was not changed (*Figure 1*) but the structure of Ca-bentonite and Na-bentonite was modified, as indicated by the change in the chemical composition, especially the SiO_2 and Al_2O_3 contents (*Figures 2* and *3*). This might be because the exchangeable cations and impurity elements in the interlayer cations were replaced by hydrogen ions (H^+) ions, after which dissolution of elements in the octahedral sheet occurred. With increasing acid concentration, the SiO_2 content increased while the Al_2O_3, Fe_2O_3, MgO, calcium oxide (CaO), sodium oxide (Na_2O), and potassium oxide (K_2O) contents decreased, possibly because leaching was likely to occur in octahedral and exchangeable cations. On treatment of Na-bentonite with various H_2SO_4 concentrations, Na minerals were increasingly eluted from the interlayer of the clay, demonstrating that calcium ions (Ca^{2+}) and magnesium ions (Mg^{2+}) were the main exchangeable cations rather than Na. Meanwhile, Ca^{2+}, potassium ions (K^+), Mg^{2+} and sodium ions (Na^+) still appeared in H_2SO_4-treated bentonite samples as exchangeable cations in the interlayer. The best activation condition was

Na-bentonite refluxed with 1.5-3.0 M H_2SO_4 solution, which correlated positively with SiO_2 and Al_2O_3 contents in the range of 74.80%-85.20% and 8.96%-13.30% by weight (wt), respectively, which was close to the composition of commercial clay.

It has been well documented that the orange-red pigmentation of degummed palm oil is mainly due to the presence of carotenoid compounds. Decolourisation of palm oil from orange-red to pale yellow improves the oil's appearance. In this study, XRF characterisation of Ca-bentonite, Na-bentonite and both activated clays revealed an association between the bleaching capacity and the SiO_2 and Al_2O_3 contents. By increasing the H_2SO_4 concentration from 0.2-5.0 M in the activation of Ca-bentonites, the SiO_2 content was increased from 71.00% to 80.00%. This clay could remove less than 30.00% of the colour, and the palm oil remained orange-red (*Figure 2*). After activation of bentonite with 5.0 M H_2SO_4, the bleaching capacity tended to increase up to 38.95%, likely due to the high acid concentration used, which induced leaching of aluminium (Al) and the elements from the interlayer cations, and the SiO_2 content increased by up to 87.30%. In addition, when the Ca-bentonites were preheated to 300°C and 500°C for 1 hr in a muffle furnace prior to refluxing with 2.0 M H_2SO_4 (F300 CBR 2.0 and F500 CBR 2.0), there was also an increase in SiO_2 content from 88.70% to 89.70%, and the Al_2O_3 content tended to decrease slightly from 7.10% to 6.55%, leading to a slightly increased bleaching capacity of 40.70%-53.33% (*Figure 2*). Clays preheated at a high temperature before activation display a higher specific surface area and total pore volume than non-preheated samples, as the clay is fully converted to an amorphous phase, which can easily leach aluminium ions (Al^{3+}) and exchangeable cations (Fernandez *et al.*, 2011; Foo *et al.*, 2011). Preheating of the Ca-bentonites at 700°C for 1 hr in a muffle furnace prior to refluxing with 2.0 M H_2SO_4 (F700 CBR 2.0), which showed a high SiO_2 content (92.30% by wt) and a low Al_2O_3 content (left only 4.28% by wt), was not favourable for the sorption of organic pigment and was ineffective for the decolourisation of palm oil (18.47% bleaching only) (*Figure 2*). This was possibly due to extensive leaching of the Al_2O_3 content, resulting in disruption or destruction of the clay sheets, which negatively affected the bleaching capacity.

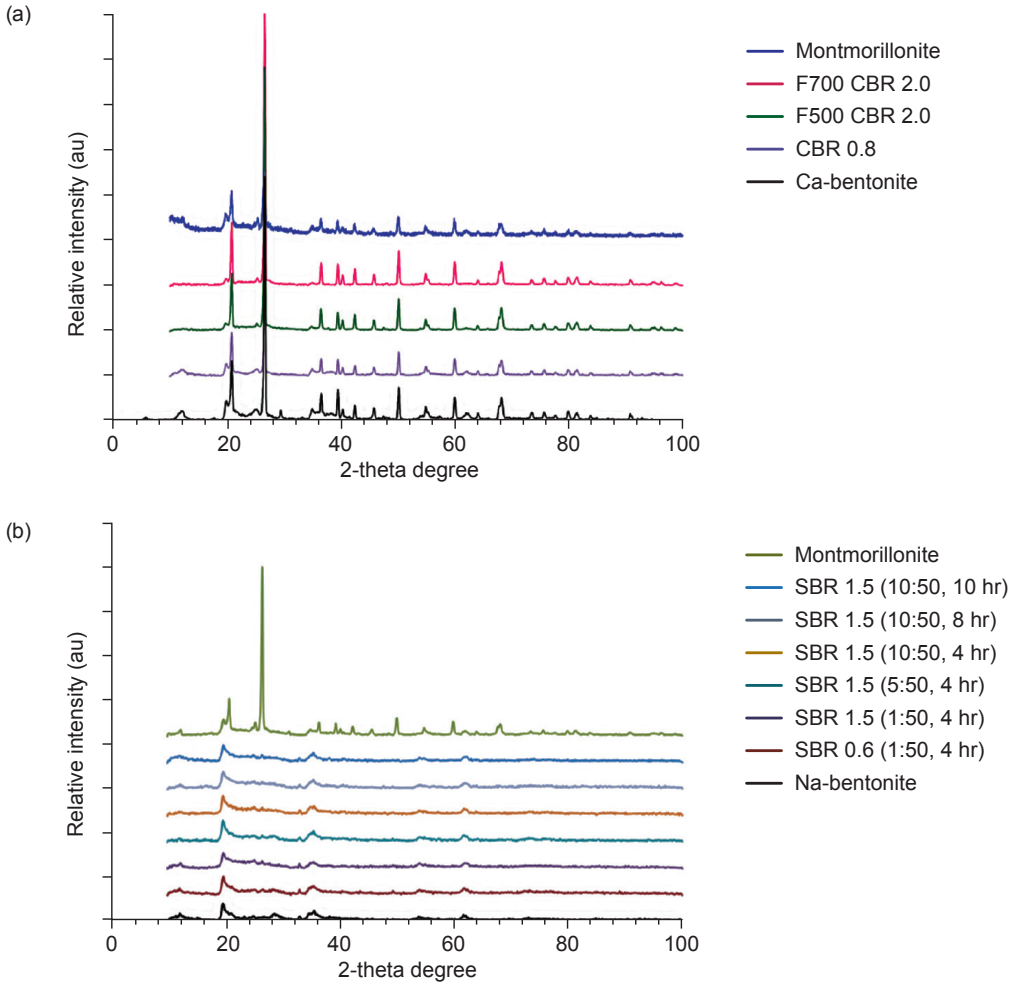
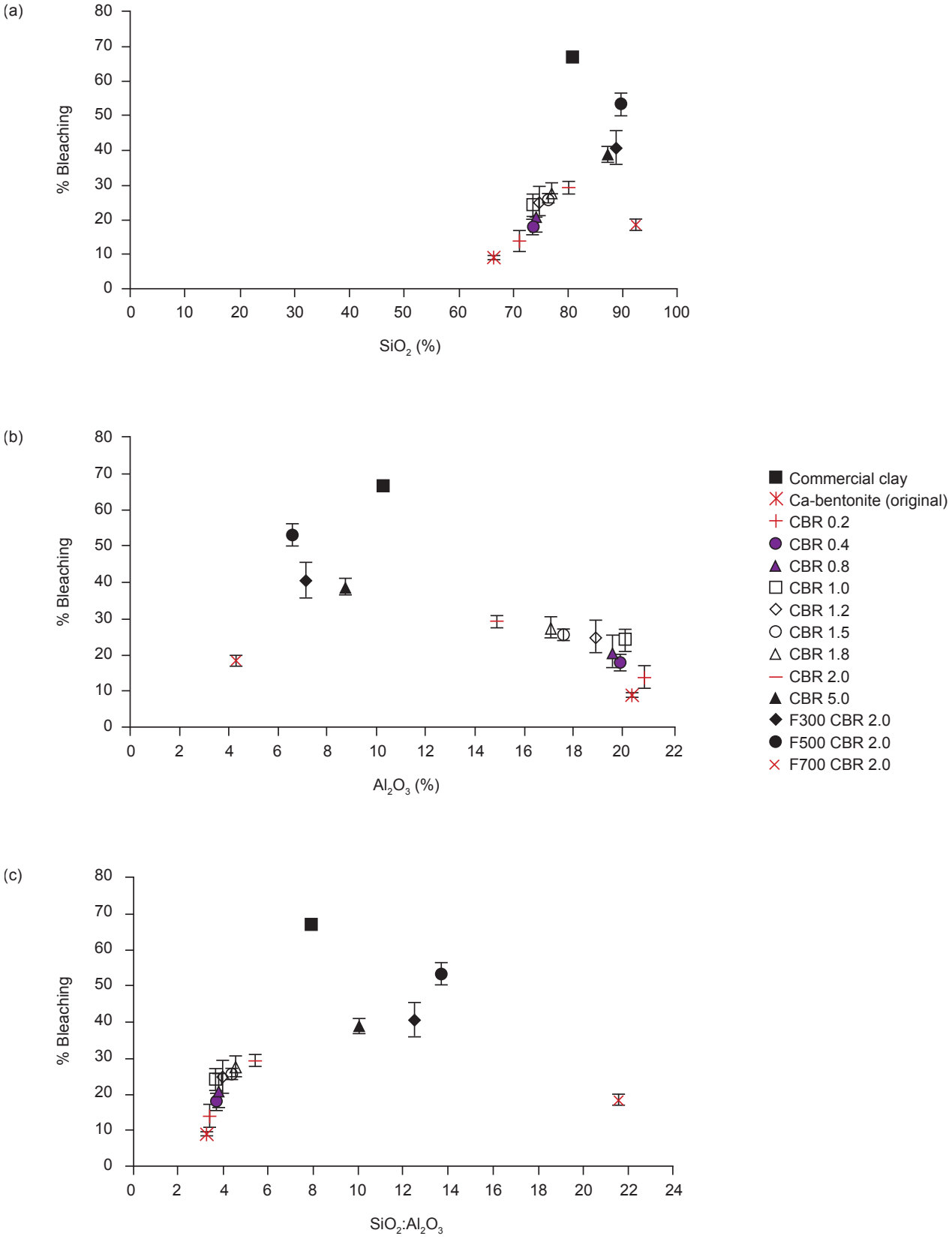
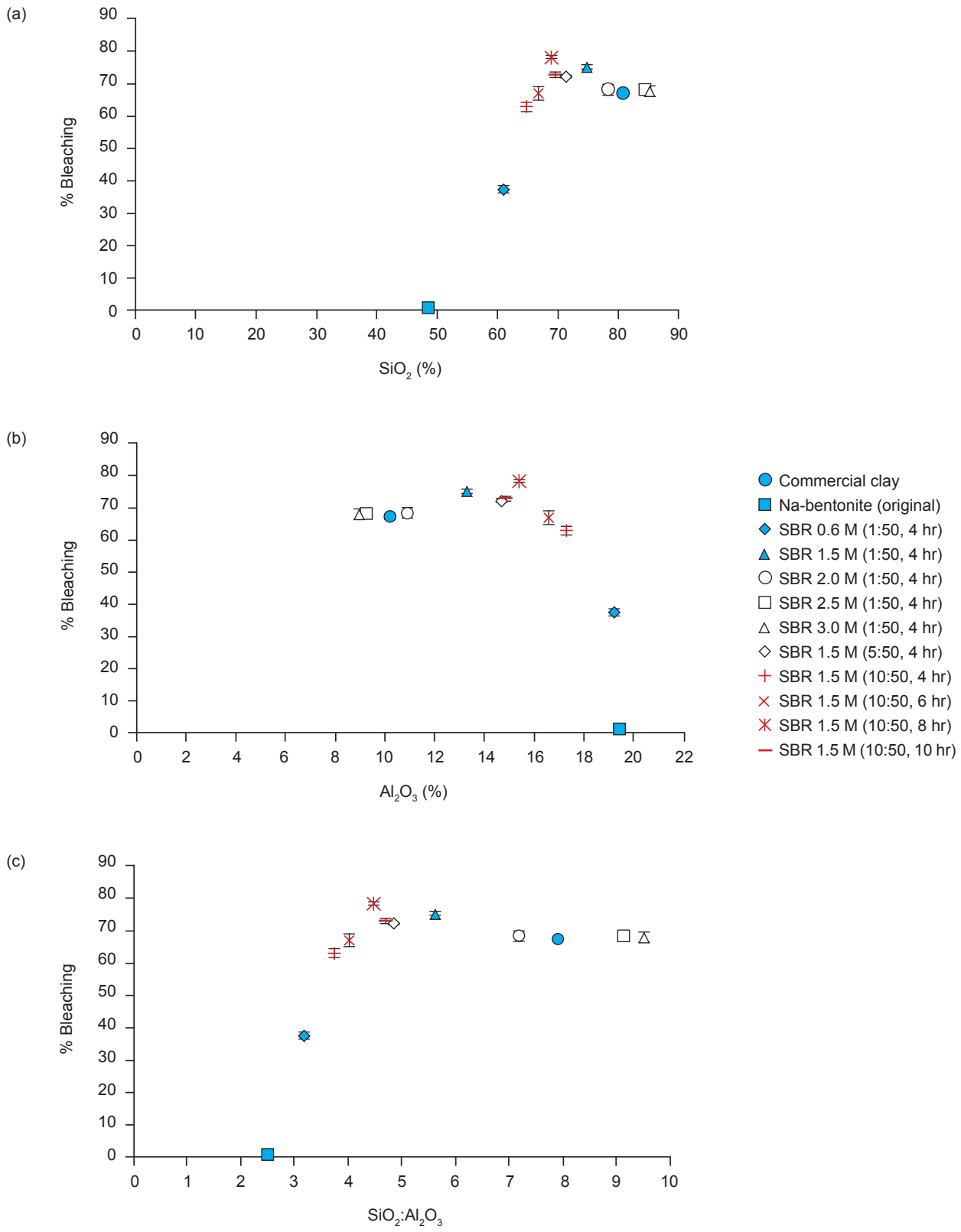


Figure 1. XRD patterns of (a) commercial clay, Ca-bentonite, and activated Ca-bentonites, and (b) Na-bentonite and activated Na-bentonites.



Note: CBR 0.2, CBR 0.4, CBR 0.8, CBR 1.0, CBR 1.2, CBR 1.5, CBR 1.8, CBR 2.0, CBR 5.0 = refluxing bentonite with H_2SO_4 solution with 0.2-5.0 M at 90°C for 4 hr and the ratio of clays to acid was 1:50 (w v^{-1}); F300 CBR 2.0, F500 CBR 2.0 and F700 CBR 2.0 = Ca-bentonites preheated at 300°C, 500°C and 700°C for 1 hr in a muffle furnace prior to refluxing with 2.0 M H_2SO_4 at 90°C for 4 hr and the ratio of clays to acid was 1:50 (w v^{-1}).

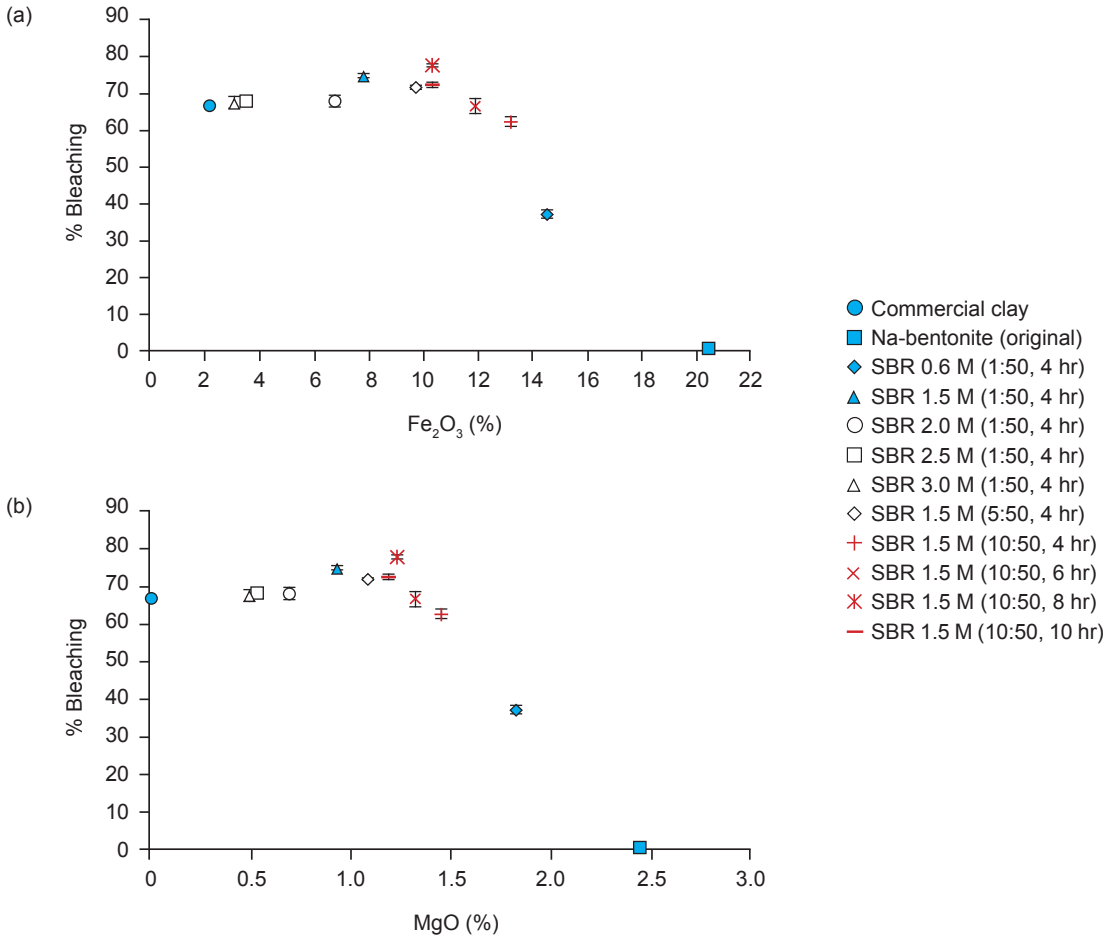
Figure 2. The relationship between % bleaching of palm oil and (a) SiO_2 contents, (b) Al_2O_3 contents and (c) the ratio of SiO_2 to Al_2O_3 contents of commercial clay, Ca-bentonite, and activated Ca-bentonite under various conditions.



Note: SBR 0.6 (1:50, 4 hr), SBR 1.5 (1:50, 4 hr), SBR 2.0 (1:50, 4 hr), SBR 2.5 (1:50, 4 hr), SBR 3.0 (1:50, 4 hr) = refluxing Na-bentonite with H_2SO_4 solution with 0.6-3.0 M at 90°C for 4 hr and the ratio of clays to acid was 1:50 (w v^{-1}). SBR 1.5 (5:50, 4 hr), SBR 1.5 (10:50, 4 hr), SBR 1.5 (10:50, 6 hr), SBR 1.5 (10:50, 8 hr), and SBR 1.5 (10:50, 10 hr) = refluxing Na-bentonite with 1.5 M H_2SO_4 concentration at 90°C for 4-10 hr and the ratio of clays to acid was 5:50 (w v^{-1}) and 10:50 (w v^{-1}), respectively.

Figure 3. The relationship between % bleaching of palm oil (a) SiO_2 contents, (b) Al_2O_3 contents and (c) the ratio of SiO_2 to Al_2O_3 contents of commercial clay, Na-bentonite, and activated Na-bentonite under various conditions.

Although other studies mentioned that activated bentonite clay had a high efficiency for pigment removal (Christidis and Kosiari, 2003; Nde *et al.*, 2019; Silva *et al.*, 2013), our results illustrated that acid-activated bentonites had a low bleaching efficiency. This might be because the properties of bentonite and activated bentonites were not suitable for decolourisation of palm oil. Interestingly, the highest bleaching capability was observed for Na-bentonite activated by 1.5 M H_2SO_4 [clay:acid ratio of 1:50 (w v^{-1}), 4 hr] containing 74.80% SiO_2 and 13.30% Al_2O_3; it could remove more colour from palm oil (75.09%) than commercial clay (67.09%). Meanwhile, the bleaching capacities of Na-bentonite activated with a high concentration of H_2SO_4 were not significantly different from those of commercial clay (68.21%, 68.32% and 67.86% for 2.0, 2.5 and 3.0 M H_2SO_4 Na-bentonite, respectively), and its SiO_2 and Al_2O_3 contents were 78.30%-85.20% and 10.90%-8.96%, respectively (*Figure 3*). Moreover, no relationship was observed between these clays and the amount of Ca, titanium (Ti), and K^+, while the Fe_2O_3 and MgO contents of activated Na-bentonites (1.5-3.0 M H_2SO_4) also influenced the bleaching efficiency of palm oil (*Figures 4a* and *4b*). On the other hand, a decline in the bleaching ability of clays was observed when a lower H_2SO_4 concentration (0.6 M) was used, which led to a different range of SiO_2 and Al_2O_3 contents (60.90% and 19.20%, respectively) (*Figures 3a* and *3b*). These results suggested that the highest bleaching of degummed palm oil was correlated with SiO_2 and Al_2O_3 contents in the range of 74.80%-85.20% and 8.96%-13.30% by wt, respectively, with a SiO_2:Al_2O_3 ratio of 5.62-9.51 (*Figure 3*). This result suggested that the amount of H^+ ions increased when the acid concentration was increased, which caused remobilisation of the tetrahedral layers of silicon ions and delamination or depletion of cations such as Al^{3+}, Mg^{2+} and Fe^{3+} from octahedral layers (Rossi *et al.*, 2011; Temuujin *et al.*, 2006) and then caused structural changes and partially decomposed the clay.



Note: SBR 0.6 (1:50, 4 hr), SBR 1.5 (1:50, 4 hr), SBR 2.0 (1:50, 4 hr), SBR 2.5 (1:50, 4 hr), SBR 3.0 (1:50, 4 hr) = refluxing Na-bentonite with H_2SO_4 solution with 0.6-3.0 M at 90°C for 4 hr and the ratio of clays to acid was 1:50 (w v^{-1}). SBR 1.5 (5:50, 4 hr), SBR 1.5 (10:50, 4 hr), SBR 1.5 (10:50, 6 hr), SBR 1.5 (10:50, 8 hr), and SBR 1.5 (10:50, 10 hr) = refluxing Na-bentonite with 1.5 M H_2SO_4 concentration at 90°C for 4-10 hr and the ratio of clays to acid was 5:50 (w v^{-1}) and 10:50 (w v^{-1}), respectively.

Figure 4. The relationship between % bleaching of palm oil (a) Fe_2O_3 contents and (b) MgO contents of commercial clay, Na-bentonite, and activated Na-bentonite under various conditions.

In addition, Lovibond analysis showed that the colour of the degummed palm oil was 49.9 red and 5.9 yellow Lovibond units, whereas that bleached with 1.5 M H_2SO_4 Na-bentonite had a colour of 16.9 red and 79.9 yellow units and was a lighter yellow than commercial clay (23.4 red and 41.8 yellow units). Therefore, although Na-bentonite clays come from different areas, the preparation of clays should give suitable SiO_2 and Al_2O_3 contents.

FFA is one of the factors that can affect oil quality. Oil bleached by commercial clay and 1.5 M H_2SO_4 Na-bentonite showed FFA (as palmitic acid) concentrations of 8.99% and 8.94%, respectively, whereas the degummed palm oil had a fatty acid content of 13.65% (*Table 1*). Presented results here on the adsorption of FFA were in agreement with those reported in a previous work (Joy *et al*., 2007), although normally FFA adsorption was found during discolouration. The adsorption of FFA of oil is described by the diffusion of FFA from oil to the active site of the clay followed by the interaction between adsorbate and the active site (Baptiste *et al*., 2020). The analysis of adsorptive properties reveals that activated Na-bentonites had a large specific surface, total pore volume and microporous, which gives them great adsorption properties (*Table 2*). Although activation of Na-bentonites could decrease their FFA content, the FFA content of activated Na-bentonites remained higher than standard specifications. For trading purposes, the maximum FFA content set by the Palm Oil Refiners Association of Malaysia in CPO is 5.00%, indicating that the use of low-quality CPO with a relatively high FFA content as the input of the bleaching process might result in a low smoke point and, consequently, high impairment of oil quality (Khor *et al*., 2019). The lengthy storage period of the CPO used in the study might have affected FFA production, thus, affecting the quality of the palm oil as a result of enzymatic lipase degradation of triglycerides. In addition, the proportion of FFA is related to the moisture content and can accelerate to rancidity levels by increasing the penetration of oxygen into the oil molecule (Hammond and White, 2011; Tan *et al*., 2017). In the presence of a high moisture content, more FFA could be generated by lipase oxidation or autocatalytic hydrolysis, resulting in rapid development of rancidity (Frank *et al*., 2011). After bleaching oil with commercial clay and 1.5 M H_2SO_4 Na-bentonite, the moisture content was below 0.10%, which could prevent an increase in FFA content (Pal *et al*., 2015).

The peroxide value is an indicator of oxidative rancidity and deterioration due to lipid peroxidation or oxidative degradation and is, consequently, an important parameter used to assess the quality of palm oil. Moreover, a high peroxide value affects the generation of free radicals, which may have adverse health impacts, such as carcinogenesis (Rossel, 1999). The peroxide value of degummed palm oil was 6.81 meq kg^{-1}, which is lower than the maximum peroxide value recommended by the Codex Alimentarius/FAO/WHO norm (10 meq kg^{-1}) (Codex Alimentarius Commission, 2011). After oil was bleached with commercial clay and 1.5 M H_2SO_4 Na-bentonite, peroxide values decreased to approximately 2.25 and 2.31 meq kg^{-1}, respectively, indicating good-quality palm oils. In addition, unsaturated fatty acids are recommended for good health over oils with highly saturated fatty acids. However, the iodine value of CPO (57.36 Wijs) is higher than the standard specification (50.00-55.00 Wijs), which indicates the ease of oxidation of the oil. Interestingly, 1.5 M H_2SO_4 Na-bentonite resulted in bleached oil having a lower iodine value (53.52 Wijs) than that obtained from commercial clay application (54.05 Wijs). Because of its anion exchange properties, activated clay often displays a high affinity for anion contaminants, including short- and long-chain alkyl carboxylates, and allows the intercalation of contaminants in the interlayer space (Celis *et al*., 2014). Moreover, modified clay can provide suitable reaction space for the dimerisation of unsaturated fatty acids and act as a catalyst, which improves dispersibility for the unsaturated fatty acid molecules to enter the interlayer (Huang *et al*., 2018). A decrease in the iodine value shows a decrease in unsaturated fatty acids, and it indicates oxidation of palm oil, representing a good quality edible oil which has a positive effect on human health (*Table 1*).

The current results showed that activation of Na-bentonites by 1.5 M H_2SO_4 could also reduce the levels of elements such as Fe, which is known to increase the rate of secondary oxidation products (Gibon *et al*., 2007). Moreover, the presence of high levels of Fe and P can contribute to changes in the flavour, colour and odour of oil. The reduction in the iron content of 1.5 M H_2SO_4 Na-bentonite was 0.78 ± 0.02 mg kg^{-1}, which is less than the maximum acceptable level stipulated in the CODEX standard (1.50 mg kg^{-1}) (Codex Alimentarius Commission, 2011), while commercial clay could reduce the iron content by approximately 1.67 ± 0.05 mg kg^{-1} (*Table 1*). The decrease in content of Fe in the bleached oils was greatest from 1.5 M H_2SO_4 Na-bentonite, possibly because it had the optimum Si and Al contents. The acid-activated clay shows availability of more adsorption sites in the matrix that are occupied by exchangeable ions in the interlayers. Furthermore, P adsorbed onto bleaching clays was removed effectively by the adsorbent 1.5 M H_2SO_4 Na-bentonite (1.94 ± 0.07 mg kg^{-1}) and commercial clay (3.13 ± 0.08 mg kg^{-1}). This information indicated that 1.5 M H_2SO_4 Na-bentonite could improve the hydrolytic stability of bleaching oil.

In addition, the most effective adsorbent for palm oil colour removal was activated Na-bentonite. The optimum activation condition was investigated

TABLE 1. PHYSICOCHEMICAL PROPERTIES OF DEGUMMED PALM OIL BEFORE AND AFTER BLEACHING WITH COMMERCIAL CLAY AND ACTIVATED Na-BENTONITES

Physicochemical property	Degummed palm oil	Commercial clay	SBR 1.5 (1:50, 4 hr)
Free fatty acid (%)	13.65 ± 0.45^{b}	8.99 ± 0.65^{a}	8.94 ± 0.31^{a}
Moisture content (g 100 g^{-1})	0.23 ± 0.10^{b}	0.04 ± 0.01^{a}	0.04 ± 0.01^{a}
Peroxide value (meq kg^{-1})	6.81 ± 0.20^{b}	2.25 ± 0.10^{a}	2.31 ± 0.10^{a}
Iodine value (Wijs)	57.36 ± 0.21^{b}	54.05 ± 0.42^{a}	53.52 ± 0.15^{a}
Iron content (mg kg^{-1})	4.13 ± 0.51^{c}	1.67 ± 0.05^{b}	0.78 ± 0.02^{a}
Phosphorus content (mg kg^{-1})	12.56 ± 0.72^{c}	3.13 ± 0.08^{b}	1.94 ± 0.07^{a}

Note: SBR 1.5 (1:50, 4 hr) = refluxing Na-bentonite with 1.5 M H_2SO_4 concentration at 90°C for 4 hr and the ratio of clays to acid was 1:50 (w v^{-1}). Data are mean ± SE (n=3); different letters in the same row indicate statistically significant differences (Anova test, Duncan, $p<0.05$).

TABLE 2. SPECIFIC SURFACE AREA, TOTAL PORE VOLUME, MICRO PORE VOLUME AND AVERAGE PORE SIZE OF COMMERCIAL CLAY AND ACTIVATED Na-BENTONITES

Sample names	Specific surface area (m^2 g^{-1})	Total pore volume (cc g^{-1})	Micro pore volume (cc g^{-1})	Average pore size (Å)
Commercial clay	160.90	0.3200	0.0900	80.47
Ca-bentonite	42.30	0.1010	0.0341	37.74
Na-bentonite	67.47	0.1256	0.0052	30.22
SBR 1.5 (1:50, 4 hr)	340.50	0.4465	0.2642	48.08
SBR 1.5 (5:50, 4 hr)	303.90	0.3780	0.2184	46..80
SBR 1.5 (10:50, 4 hr)	270.40	0.3164	0.1849	43.41
SBR 1.5 (10:50, 8 hr)	373.20	0.4672	0.2862	52.06
SBR 1.5 (10:50, 10 hr)	308.40	0.4150	0.2247	46.91

Note: SBR 1.5 (1:50, 4 hr), SBR 1.5 (5:50, 4 hr) and SBR 1.5 (10:50, 4 hr) = refluxing Na-bentonite with 1.5 M H_2SO_4 concentration at 90°C for 4 hr and the ratio of clays to acid was 1:50 (w v^{-1}), 5:50 (w v^{-1}) and 10:50 (w v^{-1}), respectively.

at a clay:acid ratio of 1:50 with 1.5 M H_2SO_4. In this experiment, a comparison of the bleaching capacity of Na-bentonite treated with 1.5 M H_2SO_4 at 90°C for 4 hr at clay:acid ratios of 1:50, 5:50 and 10:50 (w v^{-1}) was performed. The result showed that increasing the clay:acid ratio caused a decrease in the bleaching capacity (*Figure 3*). When the clay:acid ratio was increased from 1:50 to 10:50 (w v^{-1}), the structure of the clay slowly collapsed, lowering the specific surface area and total pore volume (*Table 2*). Therefore, a longer refluxing time was needed in the leaching process to investigate the most suitable conditions and SiO_2 and Al_2O_3 contents. The result showed that increasing the refluxing time from 4 hr (minimum) to 6-8 hr increased the bleaching capacity of activated Na-bentonite, but at the clay:acid ratio equal to 10:50 (w v^{-1}), the optimum time was 8 hr (78.04%). The longer time of 10 hr might have destroyed the clay structure and thus, giving a lower bleaching capacity (72.70%) than 8 hr. Therefore, the best activation condition of Na-bentonite was at H_2SO_4 concentration of 1.5 M at 90°C for 8 hr at a clay:acid ratio of 10:50 (w v^{-1}), which resulted in the clay with the highest bleaching capacity (approximately 78.04%). Alternatively, the ratio of 5:50 (w v^{-1}) and a time of 4 hr could also be applied in industry because the bleaching efficiency was only a little lower than that given by a ratio of 1:50 (w v^{-1}) (1.5 M H_2SO_4, 4 hr), but the amount of clay used in the refluxing process increased by up to five times.

CONCLUSION

The bleaching capacities were correlated with SiO_2 and Al_2O_3 contents in bleaching clay. The most suitable clay for bleaching contained SiO_2 and Al_2O_3 in the range of 68.90%-85.20% and 8.96%-16.60% by wt, respectively. This knowledge can be applied to modify other Na-bentonites from other sources to bleach palm oil efficiently. In this study, 1.5 M H_2SO_4-treated Na-bentonite was a suitable clay for bleaching palm oil. The best activation condition was Na-bentonite refluxed with 1.5 M H_2SO_4 solution at 90°C for 8 hr at a clay:acid ratio of 10:50 (w v^{-1}). Activated Na-bentonites could decolourise palm oil better than activated Ca-bentonite and commercial clay. Moreover, 1.5 M H_2SO_4 Na-bentonite was also giving the good results for the adsorption of FFA and in the elimination of peroxide, iodine, Fe, P and moisture contents. It appeared clear that activated Na-bentonite could give an attractive colour to palm oil, and also produce a stable oil of good quality.

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REFERENCES

Afolabi, R O; Orodu, O D and Efeovbokhan, V E (2017). Properties and application of Nigerian bentonite clay deposits for drilling mud formulation: Recent advances and future prospects. *Appl. Clay Sci., 143*: 39-49. DOI: 10.1016/j.clay.2017.03.009.

Aung, L L; Tertre, E; Suksabye, P; Worasith, N and Thiravetyan, P (2015). Effect of alumina content and surface area of acid-activated kaolin on bleaching of rice bran oil. *J. Am. Oil Chem. Soc., 92*: 295-304. DOI: 10.1007/s11746-015-2625-4.

Aung, L L; Tertre, E; Worasith, N; Suksabye, P and Thiravetyan, P (2014). The capacity of activated kaolins to remove colour pigments from rice bran oil: The effects of acid concentration and pre-heating prior to activation. *Clay Miner., 49*: 513-526. DOI: 10.1180/claymin.2014.049.4.02.

Ayari, F; Srasra, E and Trabelsi-Ayadi, M (2005). Characterization of bentonitic clays and their use as adsorbent. *Desalination, 185*: 391-397. DOI: 10.1016/j.desal.2005.04.046.

Baptiste, B M J; Daniele, B K; Charléne, E M; Canuala, T T L; Antoine, E and Richard, K (2020). Adsorption mechanisms of pigments and free fatty acids in the discoloration of shea butter and palm oil by an acid-activated Cameroonian smectite. *Sci. Afr., 9*: e00498. DOI: 10.1016/j.sciaf.2020.e00498.

Celis, R; Ángeles Adelino, M: Gámiz, B; Carmen Hermosín, M; Koskinen, W C and Cornejo, J (2014). Nanohybrids of Mg/Al layered double hydroxide and long-chain (C18) unsaturated fatty acid anions: Structure and sorptive properties. *Appl. Clay Sci., 96*: 81-90. DOI: 10.1016/j.clay.2014.03.026.

Christidis, G E and Kosiari, S (2003). Decolorization of vegetable oils: A study of the mechanism of adsorption of β-carotene by an acid-activated bentonite from Cyprus. *Clays Clay Miner., 51*: 327-333. DOI: 10.1346/CCMN.2003.0510309.

Codex Alimentarius Commission (2011). Codex Standards for Named Vegetable Oils, CODEX–STAN 210–1999, Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO).

Cunniff, P (1997). Official Methods of Analysis of AOAC International, 16th edition. AOAC International, Gaithersburg, Maryland.

Fernandez, R; Martirena, F and Scrivener, K L (2011). The origin of the pozzolanic activity of calcined clay minerals: A comparison between kaolinite, illite and montmorillonite. *Cem. Concr. Res., 41*: 113-122. DOI: 10.1016/j.cemconres.2010.09.013.

Firestone, D (1997). Official Methods and Recommended Practices of the American Oil Chemists' Society, 5th edition. AOCS Press, Champaign IL.

Foo, C T; Mahmood, C S and Salleh, M A M (2011). The study of aluminum loss and consequent phase transformation in heat-treated acid-leached kaolin. *Mater. Charact., 62*: 373-377. DOI: 10.1016/j.matchar.2011.01.017.

Frank, N E G; Albert, M M E; Laverdure, D E E and Paul, K (2011). Assessment of the quality of crude palm oil from smallholders in Cameroon. *J. Stored Prod. Postharvest Res., 2*: 52-58.

Gibon, V; Greyt, W De and Kellens, M (2007). Palm oil refining. *Eur. J. Lipid Sci. Technol., 109*: 315-335. DOI: 10.1002/ejlt.200600307.

Girgis, A Y (2005). Reuse of discarded deactivated bleaching earths in the bleaching of oils. *Grasas y Aceites, 56*: 34-45. DOI: 10.3989/gya.2005.v56.i1.132.

Gunawan, N S; Indraswati, N; Ju, Y H; Soetaredjo, F E; Ayucitra, A and Ismadji, S (2010). Bentonites modified with anionic and cationic surfactants for bleaching of crude palm oil. *Appl. Clay Sci., 47*: 462-464. DOI: 10.1016/j.clay.2009.11.037.

Hammond, E G and White, P J (2011). A brief history of lipid oxidation. *J. Am. Oil Chem. Soc., 88*: 891-897. DOI: 10.1007/s11746-011-1761-8.

Hassan, M S and Abdel-Khalek, N A (1998). Beneficiation and applications of an Egyptian bentonite. *Appl. Clay Sci., 13*: 99-115. DOI: 10.1016/S0169-1317(98)00021-0.

Huang, X; Yin, Y; Zhanga, B and Feng, G (2018). Effects of the structure and composition of montmorillonite on the dimerization of unsaturated fatty acids. *J. Braz. Chem. Soc., 29*: 1516-1526. DOI: 10.21577/0103-5053.20180025.

Ismail, M I; Hamidon, M H; Sofi, M Z M and Azmi, N S (2016). Renewable bleaching alternative (RBA) for palm oil refining from waste materials. *J. Appl. Environ. Biol. Sci., 6*: 52-57.

Jorhem, L (2000). Determination of metals in foods by atomic absorption spectrometry after dry dshing: NMKL collaborative study. *J. AOAC Int., 83*: 1204-1211.

Joy, N A B; Pierre, K R and Pierre, N J (2007). Adsorption of palm oil carotene and free fatty acids onto acid activated Cameroonian clays. *J. Appl. Sci., 7*: 2462-2467. DOI: 10.3923/jas.2007.2462.2467.

Kashani Motlagh, M M; Youzbashi, A A and Amiri Rigi, Z (2011). Effect of acid activation on structural and bleaching properties of a bentonite. *Iran J. Mater. Sci. Eng., 8*: 50-56.

Khor, Y P; Sim, B I; Abas, F; Lai, O M; Wang, Y; Wang, Y and Tan, C P (2019). Quality profile determination of palm olein: Potential markers for the detection of recycled cooking oils. *Int. J. Food Prop., 22*: 1172-1182. DOI: 10.1080/10942912.2019.1634098.

Lau, S Y; Phuan, S L; Danquah, M K and Acquah, C (2019). Sustainable palm oil refining using pelletized and surface-modified oil palm boiler ash (OPBA) biosorbent. *J. Clean Prod., 230*: 527-535. DOI: 10.1016/j.jclepro.2019.04.390.

Meesuk, L and Seammai, S (2010). The use of perlite to remove dark colour from repeatedly used palm oil. *ScienceAsia, 36*: 33-39. DOI: 10.2306/scienceasia1513-1874.2010.36.033.

Nde, H S; Tamfuh, P A; Clet, G; Vieillard, J; Mbognou, M T and Woumfo, E D (2019). Comparison of HCl and H_2SO_4 for the acid activation of a Cameroonian smectite soil clay: Palm oil discolouration and landfill leachate treatment. *Heliyon, 5*: e02926. DOI: 10.1016/j.heliyon.2019.e02926.

Nwabanne, J T and Ekwu, F C (2013). Decolourization of palm oil by Nigerian local clay: A study of adsorption isotherms and bleaching kinetics. *Int. J. Multidiscip. Sci. Eng., 4*: 20-25.

Pal, U S; Patra, R K; Sahoo, N R; Bakhara, C K and Panda, M K (2015). Effect of refining on quality and composition of sunflower oil. *J. Food Sci. Technol., 52*: 4613-4618. DOI: 10.1007/s13197-014-1461-0.

Rossel, J B (1999). Measurement of rancidity. *Rancidity in Foods* (Allen, J C and Hamilton, R J eds.). 3rd edition. Aspen Publishers, London. p. 22-53.

Rossi, M; Gianazza, M; Alamprese, C and Stanga, F (2003). The role of bleaching clays and synthetic silica in palm oil physical refining. *Food Chem., 82*: 291-296. DOI: 10.1016/S0308-8146(02)00551-4.

Rossi, M; Gianazza, M; Alamprese, C and Stanga, F (2011). The effect of bleaching and physical refining on color and minor components of palm oil. *J. Am. Oil Chem. Soc., 78*: 1051-1055. DOI: 10.1007/s11746-001-0387-8.

Salawudeen, T O; Dada, E O and Alagbe, S O (2007). Performance evaluation of acid treated clays for palm oil bleaching. *J. Eng. Appl. Sci., 2*: 1677-1680.

Silva, S M; Sampaio, K A; Ceriani, R; Verhé, R; Stevens, C; De Greyt, W and Meirelles, A J A (2013). Adsorption of carotenes and phosphorus from palm oil onto acid activated bleaching earth: Equilibrium, kinetics and thermodynamics. *J. Food Eng., 118*: 341-349. DOI: 10.1016/j.jfoodeng.2013.04.026.

Tan, C H; Ariffin, A A; Ghazali, H M; Tan, C P; Kuntom, A and Choo, A C Y (2017). Changes in oxidation indices and minor components of low free fatty acid and freshly extracted crude palm oils under two different storage conditions. *J. Food Sci. Technol., 54*: 1757-1764. DOI: 10.1007/s13197-017-2569-9.

Tebandeke, I Z M; Ssebuwufu, P J M; Nyanzi, S A; Schumann, A; Nyakairu, G W and Lugolobi, F (2014). Using trace metals, peroxide, acid and iodine values to characterize oils bleached using clays from central and eastern Uganda. *Am. J. Analyt. Chem., 5*: 1302-1312. DOI: 10.4236/ajac.2014.517136.

Temuujin, J; Senna, M; Jadambaa, T; Burmaa, D; Erdenechimeg, S and MacKenzie, K J D (2006). Characterization and bleaching properties of acid-leached montmorillonite. *J. Chem. Technol. Biotechnol., 81*: 688-693. DOI: 10.1002/jctb.1469.

Usman, M A; Oribayo, O and Adebayo, A A (2013). Bleaching of palm oil by activated local bentonite and kaolin clay from Afashio, Edo-Nigeria. *Chem. Process Eng. Res., 10:* 1-12.

Warasith, N and Goodman, B A (2020). Activated kaolin minerals as bleaching clays for prolonging the useful life of palm oil in industrial frying operations. *J. Am. Oil Chem. Soc., 97:* 389-396. DOI: 10.1002/aocs.12327.

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DEVELOPMENT AND VALIDATION OF AN LC-MS/MS METHOD FOR DETERMINATION OF RESIDUAL 2,4-DICHLOROPHENOXYACETIC ACID HERBICIDE IN PALM OIL

NAJWA SULAIMAN[1]*; YEOH CHEE BENG[1]; NIK SASHA KHATRINA KHAIRUDDIN[1] and FARAH KHUWAILAH AHMAD BUSTAMAM[1]

ABSTRACT

2,4-dichlorophenoxyacetic acid (2,4-D) is listed as one of the most widely used herbicides in Malaysian oil palm plantation and hence it is important to use validated methods for analysis of possible pesticide contamination in palm oil supply chain. This article reports on the development of an analytical method for the determination of residual 2,4-D in crude palm oil (CPO) using a liquid chromatography tandem mass spectrometry quadrupole time of flight (LC-QToF-MS). The method of analysis was based on a liquid-liquid extraction using methanol, heating of samples and low temperature precipitation technique. Evaluation of validation parameters such as linearity, matrix effect, selectivity, limit of detection (LOD), limit of quantification (LOQ), precision and recovery for CPO were performed. The results showed good linearity with average coefficient of determination (r^2) of more than 0.99. The LOD and LOQ for analysis of residual 2,4-D was estimated at 5.0 and 10.0 ng g^{-1}, respectively. Acceptable recoveries between 85% and 117%, repeatability with good relative standard deviation (RSD) of 5% to 10% and intermediate precision with RSD of less than 11% were obtained. Random monitoring of CPO showed that 2,4-D was not detected in any of the CPO samples.

Keywords: 2,4-dichlorophenoxyacetic acid, crude palm oil, LC-MS/MS, method validation.

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INTRODUCTION

In 2020, Malaysia produced and exported 19.14 and 4.45 million tonnes of crude palm oil (CPO), respectively (MPOB, 2020). CPO is mainly used for the production of various palm-based derivatives for both food and non-food applications such as margarine, salad dressings, tocotrienol-rich-fractions enriched chewable tablet, cream cheese, surfactants, adjuvants for cosmetics, agricultural chemicals and many other household items (Mat Dian *et al.*, 2019; Pande *et al.*, 2012; Yeong *et al.*, 2012).

[1] Malaysian Palm Oil Board,
6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.

* *Corresponding author e-mail: najwa.sulaiman@mpob.gov.my*

In Malaysia, the common weeds in oil palm plantations are *Imperata cylindrica* (lalang), *Mikania* sp., *Clidemia* sp. and *Chromolaena odorata* (Siam weed) (Jannick and Robert, 2008). These weeds compete with the oil palm trees for water, nutrients, and sunlight. This competition may affect growth and production of palm oil. Accumulation of weeds on the soil will hinder access of field operation (Jannick and Robert, 2008).

2,4-dichlorophenoxyacetic acid or 2,4-D ($C_8H_6Cl_2O_3$) is among the herbicides registered for use in Malaysian oil palm plantations, apart from glyphosate, diuron, gluphosinate ammonium and metsulphuron methyl (Malaysian Federal Government Gazette, 2020). It was reported that 2,4-D is widely used in oil palm plantation to control broad leaf weeds such as *Asystasia gangetica,*

Borreria latifolia, Cleome rutidosperma, Clidemia hirta, Diodia ocymifolia and *Mikania micrantha* (Ngah *et al.*, 2011). 2,4-D is preferable by the planters due to its good selectivity and cost-effectiveness (Mehdi *et al.*, 2011). 2,4-D may be used alone or as a mixture consisting of a combination of dicamba, metsulphuron methyl and triclopyr to control a wide range of woody and herbaceous broad leaf weeds (Murphy, 2011). This herbicide is under the phenoxy family. 2,4-D can either be in colourless crystals or colourless liquid and its melting point is 140.5°C. 2,4-D has a high solubility in water (620 mg L^{-1}) and its molecular weight is 221.0 g mol^{-1} (MacBean, 2012). This herbicide is under class II toxicity World Health Organisation (WHO) and is moderately toxic to humans, as it can cause serious eye and skin irritation (MacBean, 2012). *Figure 1* shows the chemical structure of 2,4-D.

The quality and safety of food products are always of interest to consumers and the respective authorities. This includes monitoring the presence of contaminants such as pesticide residues using validated analytical methods. From the literatures, methods for analysis of 2,4-D were described in various matrices such as eggs, milk, oranges, soil, water and olives (Chamkasem and Morris, 2016; Chen *et al.*, 2015; Garcia-Reyes *et al.*, 2007; Ismail *et al.*, 2011; Mehdi *et al.*, 2011), however none in palm oil matrix. With regard to fatty matrix, Hua *et al.* (2018) reported the use of molecular imprinted polymer surface enhanced Raman spectroscopy for detection and quantification of 2,4-D in milk, while Chen *et al.* (2018) quantified 2,4-D in rat serum using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detector and obtained average recovery of 101% with relative standard deviation (RSD) of below 8%.

The use of QuEChERS (quick, easy, cheap, effective, rugged, and safe) and solid phase extraction (SPE) technique and subsequent quantification by liquid chromatography with tandem mass spectrometry (LC-MS/MS) in negative ionisation mode was reported by Guo *et al.* (2019) and Raina and Etter (2010) for determination of 2,4-D in cereals and surface water. However, the application of liquid-liquid extraction with low temperature precipitation technique for the extraction of 2,4-D prior to quantification has not been explored, and this is the first report of such study. Combination of these techniques has the advantage of small volume of solvent needed for the extraction and it is preferred due to its simplicity and cost effectiveness. Moreover, there is no method established for the determination of 2,4-D in palm oil matrices, although 2,4-D is listed as the major herbicide used in oil palm plantation after glyphosate and metsulphuron methyl (Ainie *et al.*, 2007). As palm oil is being used as one of the key ingredients in many food products, it is important to monitor the presence of contaminants in the CPO, by developing a reliable analytical method for the analysis of 2,4-D in this matrix. Therefore, this article demonstrates a simple and economical analytical method for the determination of 2,4-D in CPO using liquid-liquid extraction and low temperature precipitation technique coupled with LC-QToF-MS application.

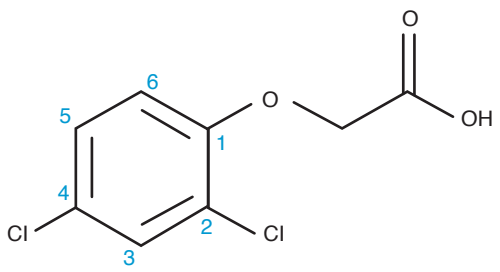

Figure 1. Chemical structure of 2,4-dichlorophenoxyacetic acid (2,4-D).

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents used in this study were of analytical or liquid chromatography grade. Methanol was obtained from Merck whereas formic acid, which is used as an additive in the mobile phase, was obtained from Fisher Scientific (USA). Deionised water from Milli-Q water purification system (Millipore Corp., USA) was used throughout the method development. 2,4-D (99% purity) standard material was purchased from Dr. Ehrenstorfer (Ausburg, Germany). All chemicals and standard solutions prepared were stored in a freezer (ProTech CF500) at the temperature of -20ºC prior to use.

CPO Samples

CPO samples were obtained from several local palm oil mills in Malaysia and subjected to drying using anhydrous sodium sulphate prior to usage. These samples were pre-tested for any residual 2,4-D to avoid interference during method development.

Apparatus

A five digit analytical balance (Sartorius SECURA 2250, Missouri, USA), ultrasonic equipment from Branson 5510 (Danbury, CT, USA), vortex mixer (model 37600 from Barnstead/Thermolyne Inc.), 2-in-1 heater and magnetic stirrer - IKA Laboratory Equipment (C-MAG HS 7), Germany and centrifugation system (Sigma

2-16P Sartorius, Missouri, USA and Thermo Scientific Heraeus Megafuge 8R, Massachusetts, USA) were used throughout the study. Adjustable micro-pipettes, between 10.0 µL to 100 µL, 100 µL to 1000 µL and 1000 µL to 5000 µL together with the pipette tips were purchased from Eppendorf (Hamburg, Germany). Consumables such as 2.0 mL capacity amber microvials (Agilent Technologies, USA), plastic pasteur pipettes, scintillation vials, self-standing Falcon tubes and test tubes were consumed in the study. Volumetric flasks with the scale of 10.0 mL, 25.0 mL, 50.0 mL and 100.0 mL (Grade A) were used for the preparation of stock and working solutions.

2,4-D Standard Solutions

2,4-D stock solution (100.0 µg mL^{-1}) was prepared in acetone by dissolving 10.10 mg of 2,4-D standard material in a 100.0 mL volumetric flask. Then, 2,4-D intermediate stock solution (10.0 µg mL^{-1} and 1.0 µg mL^{-1}) were prepared through dilution using acetone solvent.

2,4-D working standard solutions (5.0-100.0 ng mL^{-1}) were prepared by dilution of 0.05, 0.10, 0.20, 0.35, 0.50, 0.70, 0.85 and 1.00 mL intermediate stock solution (1.0 µg mL^{-1}) with acetone in each 10 mL capacity volumetric flasks. All working standard solutions were kept in the freezer at a temperature of -20ºC. 2,4-D working standards were freshly prepared on a weekly basis to be used for the analyses.

2,4-D Matrix Matched Calibration Curve

Six concentration levels of matrix matched working standard (5.0, 10.0, 20.0, 35.0, 70.0 and 100.0 ng mL^{-1} blank matrix) were prepared in blank matrix extract and injected into the LC-MS/MS system in order to construct a matrix matched calibration curve of 2,4-D for the assessment on the effect of matrix components. All analytical protocols for calibration curve establishment were conducted in triplicate.

2,4-D Spiking Samples Preparation

Spiking and extraction procedure was carried out based on modified method by Yeoh *et al.* (2019). Blank melted oil sample, 50.000 g, was weighed into a beaker and placed in a temperature controlled water bath at 60°C, with continuous stirring to ensure sample homogeneity. 0.500 mL of 2,4-D standard (1 µg mL^{-1}) was added into the oil sample to obtain final spiking concentration of 10.0 ng g^{-1}. The spiked sample was constantly stirred for 30 min to allow homogeneity. While stirring, sub-sampling of the mixed sample was carried out by weighing 5.000 g of the oil sample into eight self-standing centrifuge tubes. Several batches of spiked samples were prepared to obtain sufficient samples for method validation. These samples were stored in the freezer (-20°C) prior to usage. Similar spiking procedures were carried out for spiking levels of 50.0 ng g^{-1} and 70.0 ng g^{-1}.

Extraction of 2,4-D from Oil Samples

Melted oil sample, 5.000 g (to the nearest 0.001 g), was weighed into a self-standing Falcon tube and 5 mL methanol was added into the tube. The sample was shaken for 1 min and was left in the water bath at 60ºC for 1 min before shaking for another 1 min. The same step was repeated until a total of 5 min shaking time was achieved. This intermittent heating step during extraction is important to ensure the oil sample will not solidify and remains homogenised for higher extraction efficiency and better percentage of recovery (Khairuddin *et al.*, 2021). The sample was then centrifuged at 7000 rpm (5204 × g) for 15 min and was followed by low temperature precipitation step in the freezer (-20ºC) for 1 hr for phase separation.

After 1 hr, the sample was taken out from the freezer and an aliquot of the methanol layer (1 mL) was transferred into sample vial and shaken with vortex mixer for 3 s prior to analysis using LC-QToF-MS system. Seven replicates were prepared for each concentration levels and injected in random order to minimise instrumental drift. Recovery of 2,4-D in CPO was calculated using Equation (1):

$$\% \text{ Recovery} = \frac{\text{Amount of 2,4-D in sample}}{\text{Amount of 2,4-D added (theoretical)}} \times 100\% \quad (1)$$

Liquid Chromatography Tandem Mass Spectrometer

LC-QToF-MS analysis was conducted using AB Sciex Triple ToF (Model 5600, USA). The system was equipped with Analyst® Instrument Control version 1.7 software and MultiQuant® version 3.0.2 software. In this study, electrospray ionisation (ESI) type with negative mode was selected. Mass spectrometry (MS) parameters settings were as followed: Curtain gas, 25 psi; ion source gas (GS1), 40 psi; ion source gas 2 (GS2), 40 psi; source temperature, 450°C; and ion spray voltage, 4000 V. Kinetex C_{18} 100A capillary column (50 mm × 2.1 mm × 1.7 µm), attached to the guard column (Security Guard™ ULTRA cartridges for UHPLC C_{18}, an internal diameter of 3.0 mm) were both provided by Phenomenex™ (USA). Mobile phase A and B were 0.1% (*v*/*v*) formic acid in deionised water

and 100% methanol, respectively at the flow rate of 0.3 mL min^{-1} using a gradient system (*Table 1*). A sample of 10 µL was injected into the LC-QToF-MS system with a total analysis time of 15 min for each sample.

Determination of 2,4-D was performed via pseudo-multiple reaction monitoring (MRM) where the LC-QToF-MS is operated in MRM mode with optimised collision energy (CE) and collision energy spread (CES), in which an MRM transition is defined for each target ion. *Table 2* shows the optimised MS parameters for two ion transitions of 2,4-D. The monoisotopic mass of 2,4-D is 219.97 a.m.u. Under negative ionisation mode, formation of negative molecular ion for 2,4-D has been achieved through deprotonation with mass per charge (m/z) of 218.96. Therefore, the precursor ion for 2,4-D has been observed at m/z 218.96. As for the fragment ions (daughter ions), fragment ion with the highest signal was selected as the quantifying ion, while fragment ion with second highest signal was selected as the qualifying ion to ensure method specificity and for the confirmation of the analysis (Andrade *et al.*, 2015). *Figure 2* shows the mass spectrum of the 2,4-D fragment ions, with m/z 161 as the highest ion and m/z 125 as the second highest ion.

TABLE 1. MOBILE PHASE GRADIENT SYSTEM USED IN THE RESIDUAL 2,4-D ANALYSIS USING LC-MS/MS-QTOF

Time (min)	Flow rate (mL min^{-1})	Formic acid, 0.1% (A) (%)	Methanol (B) (%)
0.00	0.30	95	5
5.00	0.30	5	95
8.00	0.30	5	95
9.00	0.30	95	5
15.00	0.30	95	5

TABLE 2. OPTIMUM PARAMETER FOR 2,4-D OBTAINED BY MANUAL TUNING

Term used	Ion transition (m/z)	DP (V)	CE (V)	CES	Remark
2,4-D 1	218.9/160.9	-80	-20	10	Quantifying ion
2,4-D 2	218.9/124.9	-80	-20	10	Qualifying ion

Note: DP - declustering potential; CE - collision energy; CES - collision energy spread.

RESULTS AND DISCUSSION

Validation of the Developed Method

Linearity and matrix effect. Two six-point calibration curves for 2,4-D in acetone and matrix matched at different concentrations (5.0, 10.0, 20.0, 35.0, 70.0 and 100.0 ng mL^{-1}) were constructed to confirm the suitability of the chromatographic condition for this study (*Figure 3*) as well as to investigate the effect of matrix on the method performance. Upon visual evaluation, the calibration curves exhibited linear patterns with r^2 of more than 0.99 for both curves.

A big slope difference could be observed when both calibration curves were compared. The linear equations of the curves were used to calculate the matrix effect, which may exist due to the nature of the matrix and its co-eluting matrix components (Norizah *et al.*, 2020; Yeoh *et al.*, 2019). As expected,

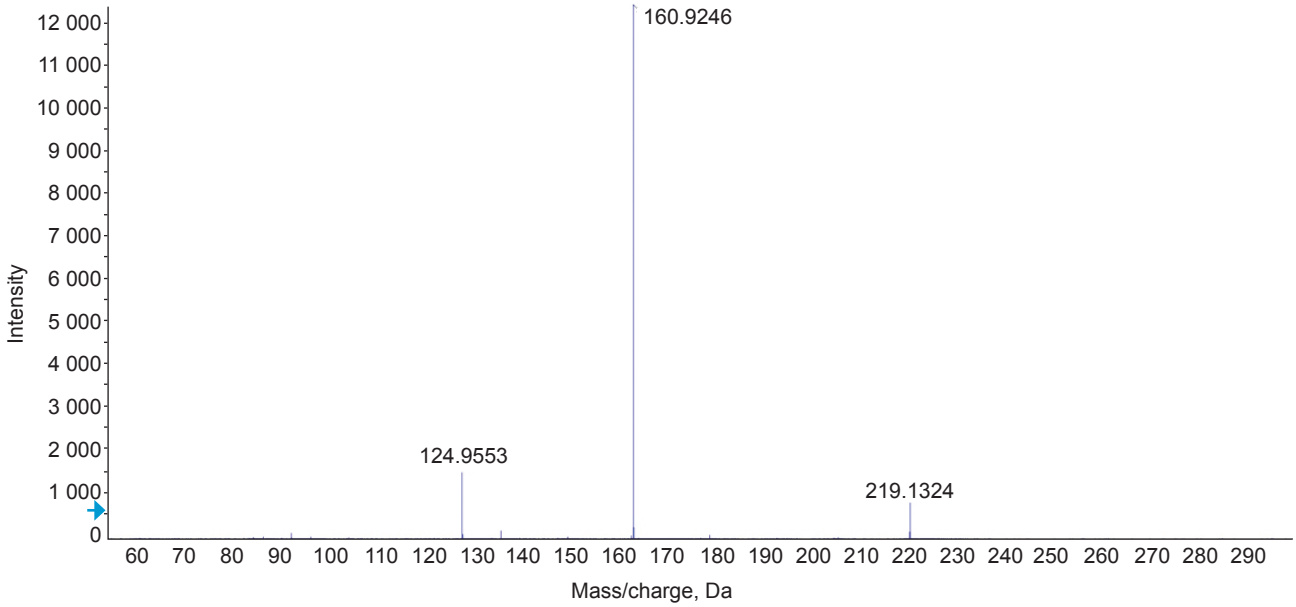

Figure 2. Mass spectrum for 2,4-D fragment ions.

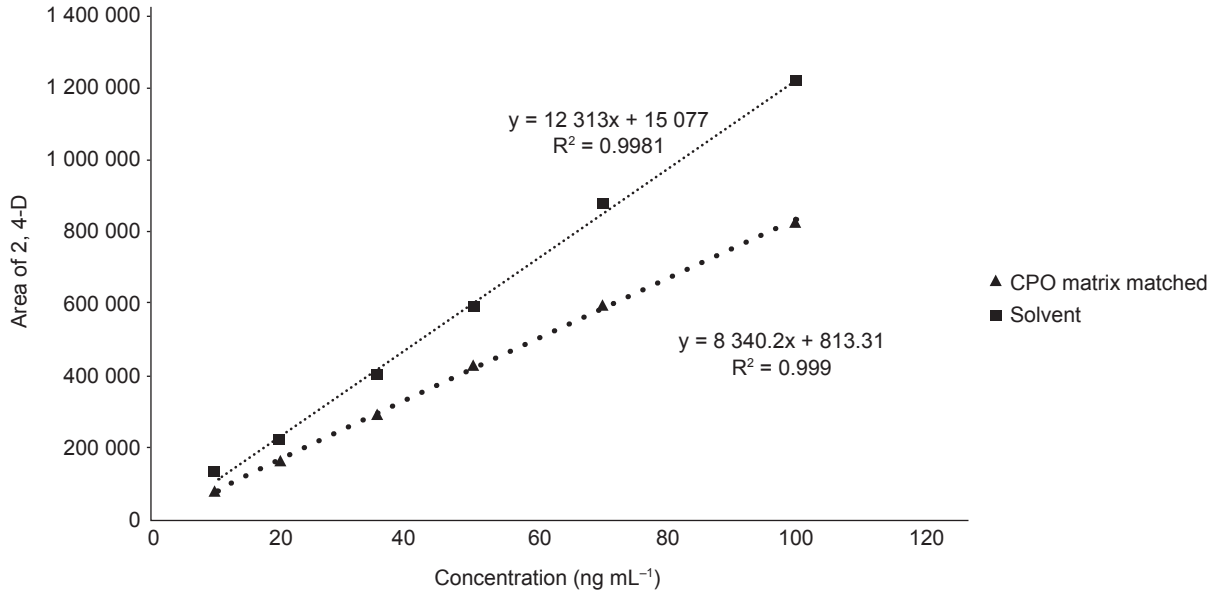

Figure 3. Comparison of calibration curve of 2,4-D plotted based on 2,4-D working standards in acetone and CPO.

the difference of slope showed that there was a significant matrix effect for the analysis of 2,4-D in CPO using LC-QToF-MS and thus, will affect the experiment results. Calculation of matrix effect was determined using Equation (2) as described by Walorczyk (2014) and Yeoh *et al.* (2019), and was found to be -46.2% showing that 2,4-D signal suffered ionisation suppression effect from the matrix.

$$\text{Matrix effect} = \left(1 - \frac{\text{Solvent slope}}{\text{Matrix matched slope}}\right) \times 100\% \qquad (2)$$

Therefore, to eliminate matrix effect, in the process of method validation, matrix matched calibration was used for quantification of 2,4-D in CPO.

Method selectivity. *Figure 4* shows the comparison of chromatograms of blank CPO and 2,4-D signal. It could be clearly seen that the signal for 2,4-D was detected at a retention time of 4.45 min with no other interfering signals, indicating satisfactory selectivity of the developed method. Selectivity of 2,4-D signal in CPO is also aided by the capability of LC-QToF-MS to perform multiple reaction monitoring on the specified precursor and fragment ions of 2,4-D.

Limit of detection (LOD) and limit of quantification (LOQ). In this work, the LOD and LOQ value for 2,4-D in CPO was determined using the calibration curve of 2,4-D against CPO matrix-matched as reported by Leito (2017). The LOD value was estimated based on the 3.3 times residual standard deviation of y of the calibration curve and 10 times of the residual standard deviation of y of the calibration curve for LOQ value.

Results from the calculation showed that the LOD and LOQ from analysis of 2,4-D in CPO was 5.1 ng g^{-1} and 15.5 ng g^{-1}, respectively. However, experimental recovery was able to quantify as low as 10.0 ng g^{-1}, with acceptable recoveries around 90% to 100%, justifiable with the current advancement of chromatography technology. Therefore, the LOD and LOQ were estimated at 5.0 ng g^{-1} and 10.0 ng g^{-1}, respectively, which are close to the value obtained from the calculation. LOD and LOQ of the analytical method is capable of detecting 2,4-D residue in CPO at levels lower than the European Union (EU) and Malaysia maximum residue limit (MRL) value for 2,4-D in palm oil which was set at 0.05 mg kg^{-1} (European Commission, 2019; Malaysian Federal Government Gazette, 2020).

Precision-Repeatability. Spiked CPO samples at concentration of 10.0, 50.0 and 70.0 ng g^{-1} were measured in one analytical run under similar operating conditions, measurement procedure, analyst and instrument. Results of the precision study were tabulated in *Table 3*. RSD obtained from different concentrations of spiked samples were much lower than acceptable limit of 20%, as prescribed in the guideline by the European Commission (2017), indicating high individual repeatability between measurement data.

Precision-Intermediate precision. Similar measurement procedure with several changes, *i.e.*, analysts, time of analysis, different batches of reagents and column efficiency were employed for spiked samples concentration of 10 ng g^{-1}. From *Table 4*, method intermediate precision for samples spiked at 10 ng g^{-1} was found to have satisfactory recoveries (70%-120%) and RSD well within

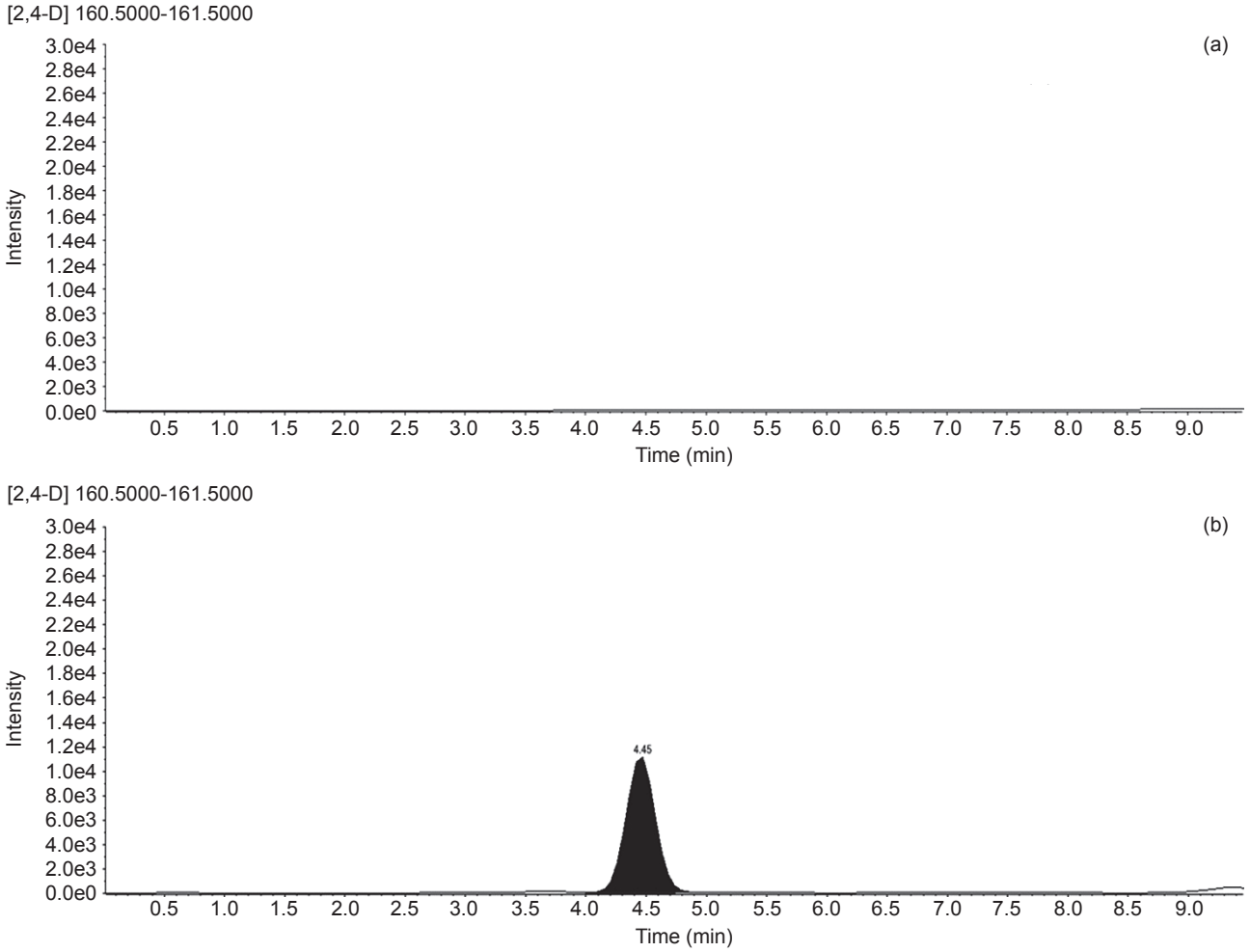

Figure 4. Representative chromatogram of (a) blank CPO sample, and (b) CPO sample spiked with 10 ng mL^{-1} 2,4-D working standard solution.

TABLE 3. RECOVERY STUDIES FOR ANALYSIS OF 2,4-D IN CPO AT THE CONCENTRATION OF 10, 50 AND 70 ng g^{-1}

Concentration (ng g^{-1})	Average recovery for analysis of residual 2,4-D in CPO (%) (n=6)	RSD (%)
10.0	117.30	5.2
50.0	88.07	9.9
70.0	85.14	8.3

TABLE 4. METHOD INTERMEDIATE PRECISION FOR SAMPLES SPIKED AT 10 ng g^{-1}

	Average recoveries (%) (n=6)		RSD (%)	
	Day I	Day II	Day I	Day II
Analyst 1	117.97	101.05	4.2	10.4
Analyst 2	116.59	114.51	5.6	6.4

acceptable criteria of 20% (European Commission, 2017). Statistical evaluation of the data supports the finding as there was no significance difference between the variation of analyst and time of analysis (p=0.01). It can be said that the developed method was reliable and reproducible (within laboratory).

Recovery experiment. Data from precision experiments (*Table 3*) were used to determine the recoveries of 2,4-D in CPO samples. The average recoveries of 2,4-D in CPO at three different concentrations (low, medium and high) were found to be between 85% to 117%, which is acceptable following the guideline stated in European Commission (2017).

Method applicability to real CPO samples. 2,4-D residue in CPO samples were evaluated using the validated method. Residual 2,4-D was not detected in randomly selected CPO samples from palm oil mills in different states in Malaysia (*Figure 5*).

CONCLUSION

A simple, economical and reliable analytical method for analysis of 2,4-D in CPO was developed and validated. Experimental results

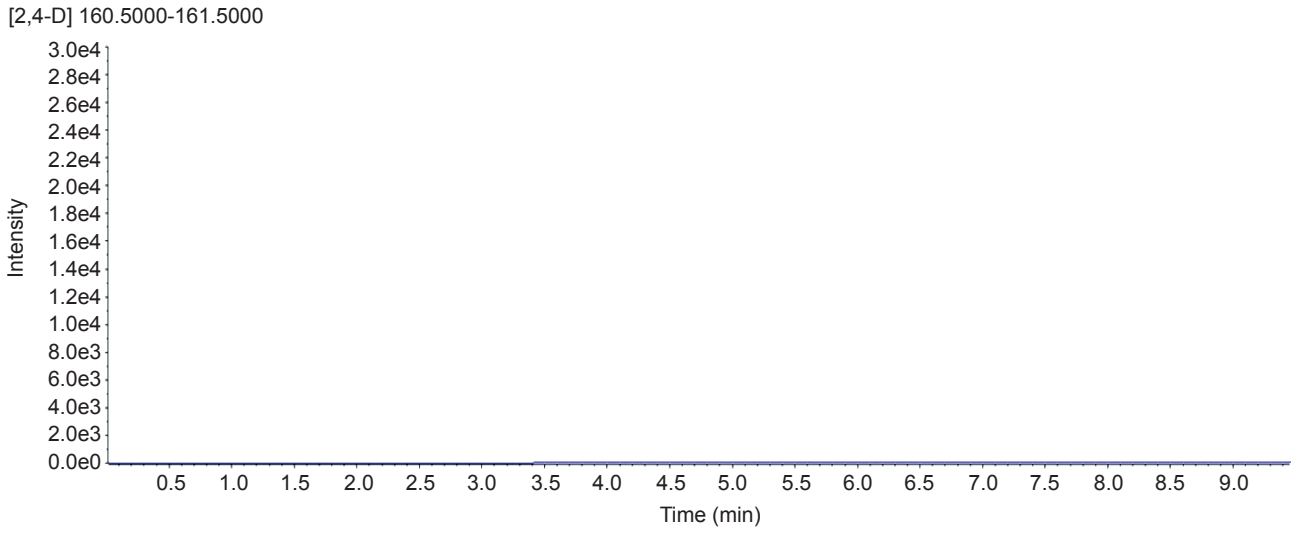

Figure 5. Example of representative chromatogram for residual analysis of 2,4-D in CPO obtained from one of the mills (Mill 1) for CPO monitoring.

revealed good method performance in terms of linearity, matrix effect, selectivity, LOD, LOQ, repeatability, intermediate precision and recovery in CPO samples. Liquid-liquid extraction with methanol, heating of samples and low temperature precipitation technique combined with LC-MS/MS were efficient in detecting 2,4-D in CPO. The LOD and LOQ of the analytical method is in compliance with the international and national MRL values set hence the method can be used for regulatory monitoring of the presence of 2,4-D residue in palm oil.

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REFERENCES

Ainie, K; Tan, Y A; Norman, K and Yeoh, C B (2007). Pesticide application in the oil palm plantation. *Oil Palm Bulletin, 54*: 52-67.

Andrade, G C R M; Monteiro, S H; Francisco, J G; Figueiredo, L A; Botelho, R G and Tornisielo, V L (2015). Liquid chromatography–electrospray ionization tandem mass spectrometry and dynamic multiple reaction monitoring method for determining multiple pesticide residues in tomato. *Food Chem., 175*: 57-65. DOI: 10.1016/j.foodchem.2014.11.105.

Chamkasem, N and Morris, C (2016). Direct determination of 2,4-dichlorophenoxyacetic acid in egg and milk by liquid chromatography/tandem mass spectrometry, Laboratory Information Bulletin LIB #4605 US Food and Drug Administration. p. 1-9.

Chen, W; Jiao, B; Su, X; Zhao, Q and Sun, D (2015). Dissipation and residue of 2,4-D in citrus under field condition. *Environ. Monit. Assess., 187(5)*: 302. DOI: 10.1007/s10661-015-4536-0.

Chen, X; Zhang, H; Wan, Y; Chen, X and Li, Y (2018). Determination of 2,4-dichlorophenoxyacetic acid (2,4-D) in rat serum for pharmacokinetic studies with a simple HPLC method. *PLoS ONE, 13(1)*: e0191149. DOI: 10.1371/journal.pone.0191149.

European Commission (2017). *Guidance Document on Analytical Quality Control and Method Validation Procedures for Pesticide Residues and Analysis in Food and Feed*. Document No. SANTE/11813/2017. 42 pp.

European Commission (2019). *EU Pesticides Database*. https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/mrls/?event=search.pr, accessed on 2 April 2021.

Garcia-Reyes, J F; Ferrer, C; Gomez-Ramos, M J; Molina-Diaz, A and Fernandez-Alba, A R (2007). Determination of pesticide residues in olive oil and olives. *Trends Anal. Chem., 26(3)*: 239-251. DOI: 10.1016/j.trac.2007.01.004.

Guo, T; Wang, X; Wang, H; Hu, Y; Zhang, S and Zhao, R (2019). Determination of phenoxy acid herbicides in cereals using high-performance liquid chromatography-tandem mass spectrometry. *J. Food Prot., 82(7)*: 1160-1165. DOI: 10.4315/0362-028X.JFP-18-558.

Hua, M Z; Feng, S; Wang, S and Lu, X (2018). Rapid detection and quantification of

2,4-dichlorophenoxyacetic acid in milk using molecularly imprinted polymers-surface-enhanced Raman spectroscopy. *Food Chem., 258*: 254-259. DOI: 10.1016/j.foodchem.2018.03.075.

Ismail, B S; Mehdi, S and Halimah, M (2011). Evaluation of herbicide pollution in the Kerian ricefields of Perak, Malaysia. *World App. Sci. J., 15(1)*: 5-13.

Jannick, J J and Robert, E P (2008). *The Encyclopedia of Fruit and Nuts*. CABI. p. 123.

Khairuddin, N S K; Yeoh, C B; Sulaiman, N; Ahmad Bustamam, F K; Halimah, M and Ismail, S (2021). Determination of metsulphuron methyl in crude palm oil using liquid chromatography triple quadrupole mass spectrometer. *Malays. J. Anal. Sci., 25(2)*: 352-362.

Leito, I (2017). Validation of liquid chromatography mass spectrometry (LC-MS) methods (Analytical Chemistry) course. https://sisu.ut.ee/sites/default/files/lcms_method_validation/files/lcms_pdf_161017.pdf, accessed on 8 October 2020.

MacBean, C (2012). 2,4-D. *A World Compendium, The Pesticide Manual, 6th Edition* (Colin MacBean ed.). British Crop Production Council, United Kingdom. p. 291-297.

Malaysian Federal Government Gazette (2020). *Food Act 1983 - Food (Amendment) (No. 3) Regulations 2020*. 38 pp.

Mat Dian, N L H; Ying, W S; Yen, F J; Meganathan, P; Nik Ibrahim, N M A; Mohd Hassim, N A; Wasoh, H and Ming, L O (2019). Palm-based vitamin E (tocotrienol-rich fraction) has excellent stability in chewable tablet after one-year of storage at ambient temperature. *J. Oil Palm Res., 31(4)*: 662-669. DOI: 10.21894/jopr.2019.0023.

Mehdi, S; Ismail, B S and Halimah, M (2011). Sorption kinetics of 2,4-dichlorophenoxyacetic acid in selected agricultural soils of Malaysia. *Res. J. Environ. Toxic., 5(1)*: 39-48. DOI: 10.3923/rjet.2011.39.48.

MPOB (2020). Malaysian Oil Palm Statistics 2020. http://bepi.mpob.gov.my/index.php/en/export/export-2020/monthly-export-of-oil-palm-products-2020.html, accessed on 2 April 2021.

Murphy, T R (2011). *Grass Pasture and Hay Field Herbicides*. Weed Science. p. 1-3.

Ngah, N; Omar, D; Juraimi, A S and Hailmi, M (2011). Leaf surface characteristics of selected Malaysian weed species of oil palm. *J. Agrobiotech., 2*: 53-65.

Norizah, H; Ainie, K; Raviraj, S and Kaushik, B (2020). High throughput residue analysis of indaziflam and its metabolites in palm oil using liquid chromatography-tandem mass spectrometry. *J. AOAC International, 103(5)*: 1237-1242. DOI: 10.1093/jaoacint/qsaa041.

Pande, G; Akoh, C C and Lai, O M (2012). Food uses of palm oil and its components. *Palm Oil: Production, Processing, Characterization and Uses* (Lai, O M; Tan, C P and Akoh, C C eds.). AOCS, United States of America. p. 571-582.

Raina, R and Etter, M L (2010). Liquid chromatography with post-column reagent addition of ammonia in methanol coupled to negative ion electrospray ionization tandem mass spectrometry for determination of phenoxyacid herbicides and their degradation products in surface water. *Anal. Chem. Insights, 5*: 1-14. DOI: 10.4137/aci.s3148.

Walorczyk, S (2014). Validation and use of a QuEChERS-based gas chromatographic–tandem mass spectrometric method for multiresidue pesticide analysis in blackcurrants including studies of matrix effects and estimation of measurement uncertainty. *Talanta, 120*: 106-113. DOI: 10.1016/j.talanta.2013.11.087.

Yeoh, C B; Farah Khuwailah, A B; Najwa, S; Nik Sasha Khatrina, K; Tay, M G and Saw, M H (2019). Development of analytical method for determination of thiosultap-disodium residue in palm oil matrix. *J. Oil Palm Res., 31(4)*: 634-640. DOI: 10.21894/jopr.2019.0028.

Yeong, S H; Idris, Z and Abu Hasan, H (2012). Palm oleochemicals in non-food applications. *Palm Oil: Production, Processing, Characterization and Uses* (Lai, O M; Tan, C P and Akoh, C C eds.). AOCS, United States of America. p. 596-620.

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EFFECTS OF INTERESTERIFIED FATS ON LIPOPROTEIN SUB FRACTIONS AND HEPATIC GENE EXPRESSIONS IN A HAMSTER MODEL

GOWRI NAGAPAN[1]*; GOH YONG MENG[2]; NOOR LIDA HABI MAT DIAN[1]; CHE ANISHAS CHE IDRIS[1]; KANGA RANI SELVADURAY[1] and NAGENDRAN BALASUNDRAM[1]

ABSTRACT

Palmitic rich interesterified (IE) fats exert detrimental effects on atherogenicity in animals but less significant effects in animal and human plasma lipids. Thus, it is important to investigate the role IE fats on lipid sub-fractions and hepatic gene expression involved in lipoprotein regulation. F1B male Golden Syrian hamsters (Mesocricetus auratus) *were fed high-fat diets* ad libitum *containing 0.1% dietary cholesterol and 30.0% energy from dietary fat, either native or IE fats namely palm olein (PO), chemically IE palm olein (CIEPO), sal fat blend (SFB) and chemically IE sal fat blend (CIESFB) for 12 weeks. Plasma lipid profiles, low density lipoprotein (LDL) and high density lipoprotein (HDL) sub-fractions and hepatic gene expression levels were determined. PO and CIEPO fed hamsters had 38% and 27% higher plasma HDL levels compared to SFB and CIESFB, respectively. Animals given PO diet had greater proportion of the larger HDL particles than SFB and CIESFB fed animals (*$p<0.05$*). Whereas, animals fed with SFB and CIESFB had greater proportion of larger LDL particles compared to both palmitic counterparts. All diets upregulated genes involved in liver fat accumulation such as CXCL16, VLDR and APO E. SFB diet showed significant (*$p<0.05$*) 16-fold upregulation in CXCL16 gene. Gene expression for ABCA1, APO A1 and CETP were upregulated all groups in response of reverse cholesterol transport (RCT). Palmitic-rich diets presented significant upregulation in APO A1 gene (*$p<0.05$*). LDL metabolism related genes such as LDLR, PCSK9, APO B, CYP7A1, PCSK9 were downregulated in all diets. In conclusion, native and IE saturated high-fat diets, induce liver steatosis in hamsters as shown in CXCL16, VLDLR and APOE expression. In this condition, cholesterol clearance via RCT was activated with expression of related genes such as ABCA1, LCAT, APO A1 and CETP. However, these effects on plasma level HDL cholesterol and large HDL sub-fractions were only seen in palmitic rich fats. Whereas, LDLR mediated cholesterol clearance was downregulated with suppression of LDLR gene with similar effects on plasma LDL in all diets.*

Keywords: hepatic genes, interesterified fats, lipoprotein sub-fractions.

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INTRODUCTION

Interesterified (IE) fats are one of the alternatives used in the food industry to reformulate and reduce the levels of *trans* fatty acids. IE fats are produced using either chemical or enzymatic method by rearrangements of the fatty acids in the glycerol backbone (Berry, 2009). The interesterification process alters the triacylglycerol (TAG) composition and physical properties of the fats and results in the repositioning of saturated fatty acids (SFA) such as palmitic or stearic acids to the *sn*-2 position which does not normally occur in most natural triglycerides. Palm oil products are excellent alternatives for *trans*-fat in food formulations. Palm oil solid fractions naturally

[1] Malaysian Palm Oil Board,
6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.

[2] Department of Veterinary Preclinical Sciences,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia, 43400 UPM Serdang,
Selangor, Malaysia.

* *Corresponding author e-mail: gowri@mpob.gov.my*

have high melting point and possess improved oxidative stability compared to other oils. *Trans*-fat replacers can be produced through established palm oil modification techniques such as blending palm oil with other oils and interesterification with different fats to alter the physical properties and produce fats with improved functionality in various food applications (Parveez *et al.,* 2020). Palm olein (PO) which has a melting point of 13°C-15°C is predominantly (65%) having monosaturated oleic acid at the *sn*-2 position. Interesterification of PO produces solid palm fat with melting point of 33°C-35°C which is more suitable for food applications as bakery fats. It has been hypothesised that the increase in SFA at the *sn*-2 position may have metabolic effects as these 2-monoacyglycerols stay intact during digestion and absorption process (Berry 2009; Filippou *et al.,* 2014).

High SFA diets have been associated with elevated serum of low-density lipoprotein cholesterol (LDL-C) levels (Sacks *et al.,* 2017), which is a risk factor for the development of atherosclerosis and cardiovascular disease (Ference *et al*., 2017). On the other hand, monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) have beneficial effects on blood lipid profiles (Mensink *et al.,* 2003; 2016). Different types of SFAs have different effects on lipid markers. Lauric, myristic and palmitic acids have plasma cholesterol raising effect while stearic acid has neutral effect (Mensink *et al.,* 2003; 2016).

Acute and chronic human trials have evaluated the effects of IE fats on cardiovascular risk and diabetes. The majority of the studies focused on the effects of IE fats on plasma lipid concentrations (Nestel *et al*., 1995; Robinson *et al.,* 2009; Sanders *et al*., 2011; Sundram *et al*., 2007; Yli-Jokipii *et al.,* 2001). In healthy individuals, palmitic acid-rich IE fat has lowered postprandial plasma triglyceride (TG) and non-esterified fatty acids (NEFA) compared to palm oil and high-oleic sunflower oil counterparts (Sanders *et al.,* 2011). A chronic human trial on hypercholesterolemic men has reported that intake of high-palm oil margarine and its IE form has similar effects on plasma lipids (Nestel *et al.,* 1995). A recent review by Alfieri *et al.* (2018) provides an overview on the role of TAG structures and IE palmitic and stearic acid-rich fats on fasting and postprandial lipemia, focusing our attention on their physical properties and their effects on human health. There are very few data regarding the effects of randomised fatty acids on fasting plasma lipemia, however, they indicate that the consumption of meals with IE fats does not influence, or is able to reduce, fasting lipemic profiles compared to consumption of meals containing a mixture of native fats with the same fatty acids profile (Alfieri *et al*., 2018).

In addition, data on the effects of randomised fatty acids on postprandial lipemia has shown the reduction of postprandial plasma TAG concentrations, in young, adult and elderly subjects, with a more pronounced effect in women compared to age-matched men. In postprandial conditions, IE fat-rich meals could be able to reduce cardiovascular diseases risk. Nevertheless, as quoted above, plasma TAG concentrations and cholesterol fractions are not the only measurable factors related to cardiovascular diseases risk, and so other independent causes still need to be evaluated (Alfieri *et al*., 2018). A recent work by Ng *et al.* (2018), has reported that IE palmitic acid diet had marginally induced adverse effects by raising body weight and body mass index (BMI) at week six and serum TAG, body fat percentage, and leptin concentrations at week 8. It was postulated that, these effects could have been due to greater fat absorption and lipogenesis in adipose tissue for the IE palm group, which suggests that the types and lengths (C16:0 and C18:0) of the fatty acids which are predominantly situated on the TAG molecule, may play an important influence on lipid metabolism (Ng *et al*., 2018).

Several animal studies have investigated the effects of these SFA at *sn*-2 position on atherosclerosis development. However, the possible mechanisms involved in this process were not explored very much. Earlier studies by Kritchevisky *et al.* (1998; 2000) have reported that the IE fat adversely influenced the severity of atherosclerosis development in male New Zealand white rabbits but did not influence cholesterol and TG plasma concentrations (Kritchevisky *et al*., 1998; 2000). Another animal study has reported that, a diet enriched with IE fats rich in SFA and PUFA showed a reduction in plasma lipids in Wistar rats, with an increase in low density lipoprotein (LDL) receptor and SREBP-2 gene expressions (Reena and Lokesh, 2007). The interesterification process did not alter plasma lipid concentrations, however, high-fat diet containing palmitic IE triggered hepatic fibrosis and adipocyte hypertrophy with inflammatory response in LDLr-KO mice (Lavrador *et al*., 2019) and induce atherosclerosis development by promoting cholesterol accumulation in LDL particles and macrophagic cells, activating the inflammatory process in LDLr-KO mice (Afonso *et al.,* 2016).

The current investigation was undertaken to look in depth at the possible effects of native and IE palmitic acid- versus stearic acid- rich fats on lipoprotein sub-fractions and hepatic gene expression in a hamster model. The effects of IE saturated fat positioning in the TAG structure on atherosclerosis and lipid profiles are well established in animal models and humans. However, the role of these type of IE fats with the SFA at the *sn*-2 position on LDL and HDL sub-fractions and hepatic gene expression particularly

on underlying mechanisms on cholesterol metabolism are limited and therefore, need further investigation.

METHODS

Hamsters and Diets

Animal procedures carried out in this study were approved by Animal Care and Use Committee, Universiti Putra Malaysia, Serdang, Selangor, Malaysia (AUP No.: R078/2014). Forty, male specific pathogen free (SPF) male Golden Syrian hamsters (*Mesocricetus auratus*), [12-14 weeks-old: 110.2 ± 9.9 g, mean ± standard deviation (SD)] obtained from Janvier laboratory, France were used for the trial. Two animals were housed per individually ventilated cage (IVC) at SPF room, at the Preclinical Research Facility, Malaysian Palm Oil Board (MPOB), Malaysia. The SPF room was maintained in a controlled environment (18°C ± 1°C, 55% humidity) with a 12 hr-light/-dark cycle. Hamsters were fed a commercial diet (Altromin #1324 Maintenance Diet) from Altromin Spezialfutter GmbH and Co. KG, Germany for two weeks. Following acclimatisation, the hamsters were randomly assigned into four groups (n=10 animals per group) and fed *ad libitum* with customised commercial high-fat diet prepared and pelleted by Altromin International, Germany for 12 weeks. The high-fat diet contained 30% energy from test fat and 0.1% cholesterol to induce the development of atherosclerosis. The nutrient compositions of the treatment diets are presented in *Table 1*. The four experimental diet groups were classified as follows: PO, chemically interesterified palm olein (CIEPO), sal fat blend (SFB) and chemically interesterified sal fat blend (CIESFB) for 12 weeks. The PUFA content namely C18:2 has been standardised in all the test fats. Slip melting point (SMP) for IE test fats have been standardised below 40°C with lower solids for better digestibility. Food intake was recorded daily, and body weights were recorded weekly.

At the end of the experiment, the hamsters were anaesthetised with a mixture of ketamine [50 mg/kg body weight (wt.)] and xylazine (10 mg/kg body wt). Blood samples were collected by cardiac puncture and plasma was separated after centrifugation at 3000 g for 15 min at 4°C and kept at -80°C until analysis. Liver samples were collected, weighed, and one part of the tissue was immediately frozen in liquid nitrogen and kept at -80°C until analysis. Liver fat extraction was a modification of the Folch method (Folch *et al.*, 1957). Approximately 3-5 g frozen liver were minced and transferred into a conical flask. Then, 50 mL of chloroform-methanol (2:1, v/v) were added, followed by a 2 min homogenisation and transferred to separating funnel. 40 mL of 0.9% (w/v) sodium chloride (NaCl) solution was added and samples were then shake for several minutes. The samples were left overnight and later the fat at the bottom layer was collected, dried, and weighed.

TABLE 1. NUTRIENT COMPOSITION OF EXPERIMENTAL DIET

Ingredients	Amount (g^{-1} kg)	% En
Experimental oils[1]	150.0	30.8
Casein	110.0	10.0
Lactalbumin	110.0	10.0
L-arginine	2.5	0.2
L-tryptophan	0.3	0.0
Corn starch	370.2	33.8
Dextrose	165.0	15.1
Cellulose	44.0	
Vitamin mix	10.0	
Mineral mix	35.0	
Choline bitartrate	2.5	
Cholesterol	1.0	

Note: [1]PO - palm olein; CIEPO - chemically interesterified palm olein; SFB - sal fat blend; CIESFB - chemically interesterified sal fat blend; customised commercial diets prepared and pelleted by Altromin International, Germany.
% En - percentage of energy.

Test Fat Preparation

PO (brand-Vesawit) was purchased from a local supermarket (Tesco Hypermarket). The CIEPO (IPO, iodine value 56), was purchased from Wilmar PGEO Edible Oils, Johor, Malaysia). Sal stearin was a generous gift from 3F Industries Hyderabad, India. Sal stearin was blended with high oleic sunflower oil in the ratio of 70:30 and subjected to chemical interesterification (CIE).

Chemical Interesterification (CIE)

CIE was performed based to the method reported by Lida *et al.* (2002). Oil was heated and dried for 30 min at 110°C under vacuum. Sodium methoxide (0.2%) was added to catalyse the reaction. After 60 min of stirring at a constant speed of 3000 rpm, the oil was then cooled to 60°C-70°C. A citric acid solution (20%) was later added to deactivate the residual catalyst. The oil was then washed with excess hot water (70°C-80°C) to remove soap by-products. Washing was repeated several times to

ensure that the sample was completely free from citric acid, catalyst residue and soaps formed by the reaction between sodium ion with any free fatty acids present or produced during the process. The IE oil was then dried under vacuum at 110°C for approximately 60 min. One percent bleaching earth was then added to the dried IE oil to lighten its colour. The oil and bleaching earth were left to react for 30 min at 90°C-100°C followed by cooling of the mixture to 60°C. The mixture was then filtered to separate the oil and the bleaching earth. The bleached IE oil was then refined to remove free fatty acids using short path distillation method at 240°C.

Determination of Fatty Acid Compositions (FAC) of Dietary Oils and Diets

The FAC of test fats was determined by converting fatty acids of TAG to fatty acid methyl esters according to AOCS Official Method (1998) with slight modification and analysed on a Perkin Elmer Autosystem GC (PerkinElmer, Inc., California, USA) using SGE Capillary BPX70 column (part No. 054602, SGE Analytical Science Pty. Ltd., Milton Keynes, United Kingdom).

From the data on the fatty-acid composition, the following were calculated: The index of atherogenicity (IA) was developed by Ulbricht and Southgate (1991), and characterises the atherogenic potential of FA. As the PUFA/SFA ratio is too general and unsuitable for assessing the atherogenicity of foods, Ulbricht and Southgate (1991) proposed a new index, IA, based on PUFA/SFA considering the available evidence, and then checked whether the resulting values were in accordance. The Equation for calculating IA is:

$$\text{Index of atherogenicity, IA} = [\text{C12: 0} + (4 \times \text{C14: 0}) + \text{C16: 0}] / \Sigma\text{UFA}$$

Determination of Fatty Acids in the *sn*-2 Position

The fatty acid esterified at the *sn*-2 position was determined according to lipase hydrolysis method using AOCS Official Method (1998). Exactly 0.5 g of sample was mixed with 100 mg of pancreatic lipase in 10 mL of tris-hydroxymethylaminomethane (Tris) buffer (pH 8.0) and mixed well. Then, 2.5 mL of bile salt (0.1% w/v) and 1.0 mL of calcium chloride (2.2% w/v) were added. The mixture was shaken well and incubated at 40°C for 1 min. One mL of hydrochloric acid and 1 mL of diethylether were then added to stop the reaction, followed by vigorous mixing by an electric shaker. Then, it was allowed to stand for separation. The bottom layer was removed, and the upper layer was spotted onto a thin-layer chromatography (TLC) plate which was then developed with hexane, diethyl ether and formic acid in proportions 70/30/1 (v/v/v). The monoacylglycerol (MAG) formed was separated using TLC plate. Plate was air dried and sprayed with 2′7′-dicholorofluorescein solution. The MAG band was identified under ultraviolet light, scraped, methylated and analysed for the FAC using a gas chromatography (GC) equipment (Agilent, Santa Clara, USA) which was equipped with a flame ionisation detector. Helium was used as the carrier gas and the total gas flow rate was at 0.8 mL min^{-1}. The oven temperature was set initially at 130°C. This temperature was then increased to 190°C at 6°C min^{-1} and later to 200°C. It was held isothermally for 2 min at 200°C.

Physico-chemical Analysis of Test Oils

The solid fat content (SFC) and SMP of the test fats were analysed by Analytical and Quality Development Unit, MPOB. The SFC and SMP of the test fats were analysed using MPOB Test Methods (Ainie *et al*., 2004). SFC was evaluated using a pulsed Nuclear Magnetic Resonance (NMR) with a non-stabilised parallel procedure (Tarmizi *et al*., 2008a). SMP was based from Tarmizi *et al.* (2008b).

Plasma Lipid Analysis

Total cholesterol (TC) and TG, high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL) analyses were conducted using enzymatic assay kits (Roche Diagnostics GmbH, Mannheim, Germany), as per manufacturers protocol on the clinical chemistry autoanalyser, Roche/Hitachi 902.

LDL and HDL Sub-fractions Analysis

Cholesterol levels in the lipoprotein sub-fractions; very low density lipoprotein (VLDL), large buoyant LDL (lbLDL), small dense LDL (sdLDL), large HDL and small dense HDL (sdHDL) were measured using the quantimetrix lipoprint LDL system, a linear polyacrylamide gel electrophoresis method (Lee *et al*., 2014). Exactly 25 µL of plasma sample was added to polyacrylamide gel tubes and followed by 200 µL of loading gel. Gel tubes were placed in the preparation rack and contents mixed by inverting the tubes for 6-8 times. Then, the gel tubes were photopolymerised for 30 min. After 30 min, the tubes were placed into the electrophoresis chamber. The top and bottom portions of the chamber were filled with electrophoresis buffer (tris-hydroxymethyl aminomethane 66.1 g/100 g, boric acid 33.9 g/100 g, pH 8.2-8.6). Electrophoresis was run for 60 min at 36 mV. Migration of the HDL fraction was observed 1 cm from the bottom of the gel tube. Gel tubes were rested for 30 min and scanned with a scanner.

Hepatic Gene Expression Analysis

Hamster liver mRNAs were isolated from liver samples and the expression levels for genes involved in cholesterol metabolism were estimated using bead-based multiplex assay as described by the manufacturer's protocol (QuantiGene; Panomics/Affymetrix, California, USA) designed for Luminex type analytical platform. Samples were analysed with a Bio-Plex 200 System Array reader with Luminex 100 X-MAP technology. Data were analysed using Bio-Plex Data Manager Software Version 5.0 (Bio-Rad). Gene expression data were normalised relative to internal control and two housekeeping genes; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and glucuronidase beta (GUSB). The QuantigenePlex 2.0 Plex Set is shown in *Table 3*.

Statistical Analyses

Statistical analyses were carried out by Graph Pad Prism software (version 6.00; Graph Pad La Jolla, CA 9203, USA). All data were checked for normality. One-way analysis of variance (ANOVA) followed by the *post-hoc* Bonferroni's multiple comparison test were performed. Value of $p<0.05$ was considered statistically significant. Data was presented as mean ± SD.

RESULTS

Test Fat Composition

The total and *sn*-2 FAC and physiochemical properties of the test fats are shown in *Table 2*. Both native and IE fat had similar total FAC and differed only in *sn*-2 FAC. The *sn*-2 proportion of palmitic acid in PO was 10.0% *vs*. 43.0% in CIEPO. As for SFB, the proportion of stearic acid was 12.0% *vs*. 39.0% in CIESFB. The PO and SFB were fully melted with lower solids of 2.3% and 1.5% at 37°C, whereas, the IE fats, CIEPO and CIESFB had 8.3% and 7.3% solids at 37°C, respectively. PO has SMP of 15°C, whereas CIEPO, SFB and CIESFB had SMP in the range of 35°C-38°C. The IA was higher in palmitic-rich test fats, IA: 0.77 compared to 0.08 in stearic-acid test fats. The IA indicates that the relationship between the sum of SFAs and the sum of unsaturated fatty acids (UFAs). The main classes of SFAs, which include C12:0, C14:0 and C16:0, with the exclusion of C18:0, are considered pro-atherogenic (they favour the adhesion of lipids to cells of the circulatory and immunological systems. UFAs are considered to be anti-atherogenic as they inhibit the accumulation of plaque and reduce the levels of phospholipids, cholesterol, and esterified fatty acids. Therefore, the consumption of foods or products with a lower IA can reduce the levels of TC and LDL-C in human blood plasma (Chen and Liu., 2020).

Food Intake and Body Weight

The total food intake, weight changes and weekly body weights are as shown in *Table 4*. All groups showed body weight losses with highest in PO followed by SFB, CIESFB and CIEPO groups. CIEPO group showed the lowest body weight loss. However, the results were not statistically significant. Food intake was highest in SFB followed by CIESFB, CIEPO and PO. However, no significant difference was noted. Liver weight was significantly lower in CIESFB compared to PO and CIEPO groups.

TABLE 2. FATTY ACID COMPOSITION, SFC, SMP AND IA OF THE TEST OILS

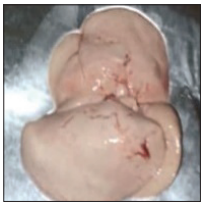
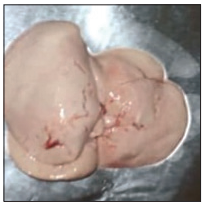
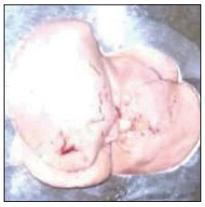
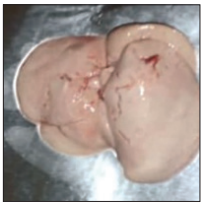
FAC (%)	PO		CIEPO		SFB		CIESFB	
	sn-1,2,3	*sn*-2	*sn*-1,2,3	*sn*-2	*sn*-1,2,3	*sn*-2	*sn*-1,2,3	*sn*-2
C16:0	39.9	10.2	41.0	43.0	4.6	2.4	4.6	5.22
C18:0	4.4	1.6	4.3	4.2	34.0	12.2	35.0	38.8
C18:1	44.1	67.0	43.2	41.0	45.0	78.5	45.0	46.8
C18:2	10.4	19.0	9.9	10.0	10.5	5.0	10.4	3.1
C20:0	0.3	0.1	0.3	0.3	4.1	2.7	4.6	5.2
C18:3	0.2	0.3	0.1	0.1	0.2	0.2	0.2	0.1
SMP	15.3°C		35.4°C		35.1°C		38.3°C	
SFC	2.3		8.3		1.5		7.3	
Index of atherogenicity (IA)	0.73		0.77		0.08		0.08	

Note: FAC - fatty acid composition; IA - index of atherogenicity; SFC - solid fat content; SMP - slip melting point; PO - palm olein; CIEPO - chemically IE palm olein; SFB - sal fat blend; CIESFB - chemically IE sal fat blend.

TABLE 3. GENES OF INTEREST FOR HEPATIC GENE EXPRESSION ANALYSIS

Genes	Abbreviations	Accession numbers	Species
Chemokine (C-X-C motif) ligand 16	CXCL16	XM_005067827	Golden hamster
Low-density lipoprotein receptor isoform X2	LDLR	XM_005078537	Golden hamster
Stabilin 1	STAB1	XM_013111841	Golden hamster
Apolipoprotein E	APOE	XM_005086320	Golden hamster
Very low-density lipoprotein receptor	VLDLR	XM_013110512	Golden hamster
Proprotein convertase subtilisin/kexin type 9	PCSK9	XM_013114871	Golden hamster
Apolipoprotein A1	APOA1	NM_001281657	Golden hamster
Apolipoprotein B-100	APOB	XM_005079084	Golden hamster
ATP-binding cassette sub-family A member 1	ABCA1	XM_005076485	Golden hamster
Phosphatidylcholine-sterol acyltransferase	LCAT	XM_005076352	Golden hamster
Apolipoprotein D	APOD	XM_003495384	Chinese hamster
Scavenger receptor class B member 1 isoform X4	SCARB1 (SRB1)	XM_013120965	Golden hamster
Cholesteryl ester transfer protein	CETP	M63992	Golden hamster
Cholesterol 7-alpha-monooxygenase	Cyp7a1	XM_016972392	Chinese hamster
Acetyl-CoA acetyltransferase 1	ACAT1	XM_005069541	Golden hamster
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	U10983	Golden hamster
Hypoxanthine-guanine phosphoribosyltransferase	HPRT	XM_005085546	Golden hamster
Glucuronidase, beta	GUSB	XM_013120748	Golden Hamster

TABLE 4. GROWTH PARAMETERS IN HAMSTERS AFTER INTAKE OF EXPERIMENTAL DIETS FOR 12 WEEKS

Group	PO	CIEPO	SFB	CIESFB
Weight changes (g)	-16.94 ± 7.14	-12.56 ± 7.75	-16.58 ± 10.21	-16.39 ± 4.92
Total food intake (g)	889.77 ± 186.48	862.52 ± 171.92	984.37 ± 232.49	892.53 ± 195.35
Liver				
Liver weight (g)	10.33 ± 0.92a	10.25 ± 1.09a	9.33 ± 1.73	8.30 ± 0.91b
Liver fat content (per 100 g)	19.41 ± 7.62	23.98 ± 1.84	19.47 ± 5.75	22.93 ± 3.24

Note: PO - palm olein; CIEPO - chemically IE palm olein; SFB - sal fat blend; CIESFB - chemically IE sal fat blend.

TABLE 5. PLASMA LIPIDS, HDL AND LDL SUB-FRACTIONS

Parameters	PO	CIEPO	SFB	CIESFB
Lipid profile (mg/dL)				
TC	487.66 ± 76.69	456.3 ± 83.4	426.03 ± 53.23	435.89 ± 50.57
TG	116.8 ± 29.64	104.3 ± 24.91	98.08 ± 24.49	91.40 ± 29.96
LDL	222.53 ± 96.52	204.67 ± 56.99	226.82 ± 71.99	228.21 ± 54.2
HDL	292.66 ± 16.52a	268.61 ± 40.78a	230.41 ± 34.02b	255.23 ± 26.53b
TC/HDL ratio	1.67 ± 0.28	1.71 ± 0.27	1.89 ± 0.39	1.72 ± 0.20
LDL sub-fractions (%)				
VLDL	14.53 ± 2.47	15.46 ± 1.55	15.22 ± 2.04	15.84 ± 0.53
Large LDL	8.98 ± 3.33a	9.97 ± 3.84a	15.08 ± 4.63b	13.19 ± 3.26b
sd LDL	1.45 ± 2.02	0.91 ± 0.56	2.24 ± 2.03	1.79 ± 1.55
Mean LDL size (nm)	268.6 ± 4.427	269 ± 3.33	268.8 ± 4.71	268.4 ± 4.4
HDL sub-fractions (%)				
Large HDL	44.9 ± 7.58a	43.27 ± 3.42ab	36.95 ± 5.79b	36.07 ± 4.26bc
sd HDL	1.77 ± 0.88	2.19 ± 1.21	2.83 ± 0.92	2.26 ± 1.36

Note: HDL - high density lipoprotein; LDL - low density lipoprotein; sd HDL - small dense high-density lipoprotein; sd LDL - small dense low-density lipoprotein; TC - total cholesterol; TG - triacylglycerol; VLDL - very low-density lipoprotein; PO - palm olein; CIEPO - chemically IE palm olein; SFB - sal fat blend; CIESFB - chemically IE sal fat blend.

n=10, Values are means ± SD; Means with unlike alphabets indicate a significant difference of $p \leq 0.05$.

Plasma Lipids, LDL and HDL Sub-fractions

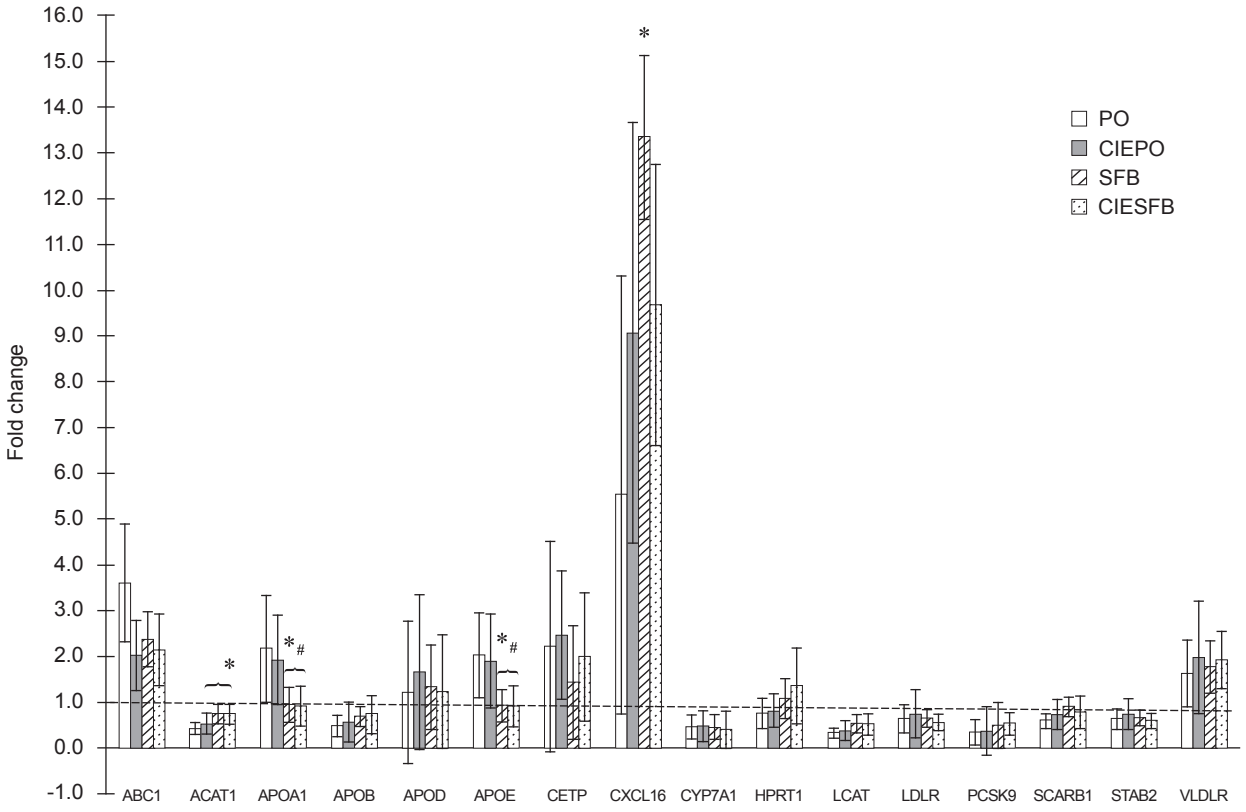
Plasma lipid profile, LDL and HDL sub-fractions are as shown in *Table 5*. The PO group presented greater HDL cholesterol compared to SFB and CIESFB groups. Other lipid parameters were similar in all groups.

Animals fed with PO and CIEPO diets had increased proportion of larger HDL particles compared to stearic acid rich native and IE fats fed animals (p<0.05). Plasma small dense HDL sub-fractions were similar in all groups. Large LDL sub-fractions were found increased in SFB and CIESFB fed animals compared to palmitic rich fat groups. However, the interesterification processes did not show any significant impact in all these lipoprotein particles levels.

Effects of *sn*-2 Palmitic *vs*. Stearic Acid on Hepatic Gene Expressions

Hepatic gene expression levels are depicted in *Figure 1*. Genes involved in cholesterol metabolism such Chemokine (C-X-C motif) ligand 16 (CXCL16), ATP-binding cassette sub-family A member 1 (ABCA1), apolipoprotein A (APO A1), apolipoprotein E (APOE), cholesteryl ester transfer protein (CETP) and very low-density lipoprotein receptor (VLDLR) were upregulated. CXCL16 gene was found highly expressed all diets and significant differences seen (13 fold, p<0.05) in SFB compared to PO. CXCL16 is often involved in inflammation-mediated lipid accumulation in liver. This finding was confirmed with signs of hepatic steatosis which was observed in all groups as shown in *Figure 2*. Significant group differences found in other genes such as APOE (PO *vs.* SFB, CIESFB and CIEPO *vs.* SFB, CIESFB p<0.05); APO A1 (PO *vs.* SFB, CIESFB and CIEPO *vs.* SFB, CIESFB, p<0.05).

Gene expression of acetyl-CoA acetyltransferase 1 (ACAT), apolipoprotein B (APOB), phosphatidylcholine-sterol acyltransferase (LCAT), low-density lipoprotein receptor isoform X2 (LDLR), proprotein convertase subtilisin/kexin type 9 (PCSK9), scavenger receptor class B member 1 isoform X4 (SCARB/SRB1) and stabilin 1 (STAB1) were down-regulated showing lower fold change than 1 with no group effects. Significant group differences were found in expressions of ACAT (PO *vs.* SFB, CIESFB p<0.05).



Note: The gene expression data was normalised to two housekeeping genes (GAPDH, GUSB). The genes fold change was calculated by dividing the normalised value for the experimental samples PO - palm olein; CIEPO - chemically IE palm olein; SFB - sal fat blend; CIESFB - chemically IE sal fat blend by the normalised value of internal control. n=10, values are means ± SD; * p<0.05 *vs.* PO group; # p<0.05 *vs.* CIEPO group.

Figure 1. Hepatic gene expression.

DISCUSSION

The main objective of this study was to evaluate the effects of IE palmitic and stearic-rich high-fat diets with added cholesterol on lipid profiles, lipoprotein sub-fractions and hepatic gene expression in hamster model. From the obtained results, 12 weeks high-fat diets with added cholesterol intake resulted in upregulation CXCL16 gene in all diets with 13 fold significant upregulation in SFB group in comparison with PO group. CXCL16 gene expression is often associated with fatty liver which was observed as pinkish liver in the hamsters. It has been reported that CXCL16 is involved in the pathogenesis of inflammatory diseases, such as atherosclerosis, cancer and kidney diseases (Barlic *et al*., 2009). In our study, both saturated fats in native and IE form in high-fat scenario may have detrimental effects as seen in the gene expression levels of CXCL16 which was significantly increased causing inflammatory stress suggesting that the CXCL16 pathway may be involved in the development of fatty liver in hamsters.

Beside to the high expression of CXCL16, another gene often associated to fatty liver is VLDLR. VDLR is a member of the LDLR family and is involved in lipid transport via apolipoprotein recognition. In VLDLR-deficient mice, blood TG concentration is high and increased lipid uptake into adipose tissue and demonstrating that VLDLR has a function in the uptake of TG-rich VLDL into peripheral tissues (Oshio *et al*., 2021). VLDLR is highly expressed in tissues with active lipid metabolism, such as the heart, skeletal muscle, and adipose tissue (Tiebel *et al*., 1999). The expression of VLDLR in the liver has been demonstrated to be lower than that in other organs. However, hepatic VLDLR gene expression is induced during endoplasmic reticulum (ER) stress, as well as fatty liver, and both of these inductions disappear in VLDR-deficient and APO-E deficient mice (Jo *et al.*, 2013). We found that all high-fat diets induce upregulation of VLDLR and APOE it is strongly suggested that VLDLR-mediated lipid uptake into the liver may be a major cause of hepatic fat accumulation during ER stress. However, plasma TG levels were not affected in this study.

We found that both native and IE oils, namely PO and CIEPO diets beneficially elevated plasma HDL cholesterol levels regardless of the positioning of palmitic acid in TAG backbone compared to the stearic acid rich diets. HDL is the smallest lipoprotein involved in scavenging of excess cholesterol from peripheral tissues to liver via RCT. We next quantified the HDL sub-fractions by the Lipoprint gel electrophoresis system. Larger HDL particles were found to be increased in PO and CIEPO diets compared to stearic acid-rich diets.

The increases in both plasma HDL and HDL particle size in PO and CIEPO diets, were further confirmed with the expression of ABCA1 and CETP genes. Intake of palmitic and stearic- rich SFA diets resulted in upregulation of ABCA1 and CETP genes in liver. These observations may be due to the high cholesterol loading from the diets in which excess cholesterol is removed from arterial cells and sent to the liver for excretion into the bile acid via RCT which is mediated by HDL (Zelcer and Tontonoz, 2006). RCT promotes removal of excess cholesterol from arterial wall macrophages to the liver for conversion to bile acids and subsequent excretion in faeces (Zelcer and Tontonoz, 2006). A study has evaluated the effects of saturated fat on RCT in mice with similar results in HDL levels, but in this case the authors did not find differences in the rate of macrophage RCT (Escolà-Gil *et al*., 2011; O'Reilly *et al*., 2016). HDL confers atheroprotection by protecting arterial wall from atherosclerotic plaques formation (Borén and Williams, 2016). APO A1 (70% of the protein content of HDL) interacts with ABCA1 receptors in various cell types (hepatocyte, enterocytes and macrophages) to remove excess phospholipids and cholesterol to APO A1. This process results in the formation of nascent HDL particles (pre-β HDL), which can subsequently interact with Scavenger receptor class B member 1 (SR-B1) and ATP-binding cassette, sub-family G, member 1 (ABCG1), with the purpose of incorporating more cholesterol, forming a mature molecule of HDL (α-HDL). These processes are catalysed by the enzyme Lecithin-cholesterol acyltransferase (LCAT) (Marques *et al*., 2018; Shen *et al*., 2018). LCAT deficiency is associated with severely reduced concentrations of HDL and APO A1, whereas transgenic animals overexpressing LCAT show markedly higher plasma HDL and APO A1 levels (Huang *et al*., 2016). In the present study, LCAT activity in hamsters fed both palmitic and stearic-rich diets were found suppressed. A number of studies showed a relationship of LCAT expression with the expression of APO A1. Incubation of hepatocytes with cytokines or injection of endotoxin in rats simultaneously reduced the plasma concentration of APO A1 and LCAT activity. On the other hand, there is no reduction in hepatic APO A1 mRNA (Rudling *et al*., 2002), suggesting that the two genes may not be coordinately regulated. Plasma HDL cholesterol concentration is positively associated with the concentration of APO A1; thus, APO A1 gene expression may be an important determinant of HDL cholesterol levels (Getz and Reardon, 2017). In humans, SFA rises APO A1 protein and HDL cholesterol concentration, while PUFA decreased HDL cholesterol concentrations (Dorfman *et al.*, 2005; Hatahet *et al.*, 2003; Lichtenstein *et al*., 1999; Plump, 1994). In contrast to the previous

studies, our findings showed that hepatic APO A1 gene expression was higher in both palmitic rich diets with concomitant increase in plasma HDL cholesterol and large HDL sub-fractions with suppression of LCAT gene. This finding is in agreement to an earlier work which reported SFA intake lowered LCAT activity in hamsters (Dorfman *et al.*, 2005; Fungwe *et al.*, 1998).

Scavenger receptor class B type I (SRB1) gene was found downregulated in palmitic-rich diets which maybe became responsible for the elevation of plasma HDL and their sub-fractions particularly the large HDL particles. Hepatic SRBI is an HDL receptor that plays a role in determining and circulating levels of HDL. Overexpression of SRB1 is associated with lower concentrations of HDL cholesterol and associated with an increased risk of CVD (Fungwe *et al.*, 1998; Spady *et al*., 1999). SRBI increases HDL mediated transport of cholesteryl ester from peripheral tissues to the liver and reducing the cholesteryl ester transfer protein (CETP)-mediated transfer of cholesteryl ester to apoB-containing lipoproteins. It was reported that, hepatic mRNA levels of SRB1 were elevated in hamsters fed PUFA diets compared to SFA (butter and coconut oil) and *trans* fatty acids (Dorfman *et al.*, 2005). Dietary fatty acids have an impact on SRB1 which regulates the circulatory pool of cholesterol. Although SFA-rich diet has beneficial effects on HDL cholesterol concentrations, palmitic acid rich fats might have more beneficial effects compared to stearic acid- rich diets in the metabolism of HDL. CETP facilitates the exchange of cholesteryl ester and TG between lipoproteins from HDL through action of LCAT to LDL which subsequently contributes to the plasma LDL levels. Although CETP gene was found upregulated in all diets, however the LCAT expression was found downregulated with no effects seen on the plasma LDL levels in all diets.

With regards to LDL metabolism, it was found that intake of both stearic-rich diets had elevated large LDL particles with the downregulation of LDLR gene. LDLR found in liver is responsible for the removal of plasma LDL and lowers plasma cholesterol concentrations. LDLR is an important marker of atherosclerosis (Haerer *et al*., 2012; Notarnicola *et al*., 2010). Human studies have also shown that nearly 60%-70% of the excess plasma cholesterol is transported via LDL-receptor mediated uptake and HDL-mediated RCT (Goldstein and Brown, 2009; Han *et al.*, 2012). Under normal conditions, LDL is removed from circulation mostly by LDLR mediated liver uptake so that LDL cholesterol levels are stable in plasma. The absence of LDLR causes LDL to accumulate in plasma and removal is totally disrupted. Increased level of LDLR expression will result in reduction of serum LDL cholesterol levels by enhancing the uptake and removal of LDL cholesterol (Reena *et al.*, 2011). In this study, high-fat diet induced suppression in the LDL receptor, which mediated clearance of LDL particles in all diets in hamsters.

As shown in previous studies (Afonso *et al.*, 2016; Kritchevsky *et al*., 1998; 2000), positioning of palmitic and stearic in *sn*-2 position with interesterification process did not modify the plasma lipid concentrations, as seen between PO *vs.* CIEPO and SFB *vs.* CIESFB diets fed hamsters. Both SFAs showed similar effects in lipids profiles namely TC, LDL, TC/HDL ratio and TG. Further analysis with LDL sub-fractions, showed that stearic acid rich diets have large LDL particles. These findings are in agreement with a recent study in LDLr-KO mice by Afonso *et al.* (2016). Both palmitic and stearic acid diets had normal patterns of LDL and HDL sub-fractions, consisting of primarily larger and buoyant with less small dense particles.

CONCLUSION

Our findings suggest that, native and IE saturated high-fat diets, had induced liver steatosis in hamsters as shown from the expression of CXCL16, VLDLR and APOE genes that induced hepatic fat accumulation. In this condition, cholesterol clearance via RCT was activated with expression of related genes such as ABCA1, LCAT, APO A1 and CETP. However, these effects on plasma levels of HDL cholesterol and large HDL sub-fractions were only observed in hamsters fed with palmitic rich fats. Whereas LDLR mediated cholesterol clearance was downregulated with suppression of LDLR gene, with similar effects on plasma LDL in all diets.

The Limitations of the Study

There are some limitations of the present study which should be noted. For instance, although hepatic steatosis was observed, no further histological, markers of liver injury and inflammation analysis were conducted to identify the severity of the hepatic steatosis induced by the tested diets. Therefore, further studies are warranted in this context. Besides that, plasma levels of CETP and LCAT were not determined in this study. The strength of our study relies on the use of hamsters as a model, which is widely accepted as a suitable animal model for studying human cholesterol metabolism. The lipid profiles and susceptibility to dietary cholesterol of the golden Syrian hamster (*Mesocricetus auratus*) are similar to those of human (Kris-Etherton and Dietschy, 1997). In human and the hamster, LDL is the dominant lipoprotein, whereas HDL is the major plasma lipoprotein in other animal models

such as mouse, rat and monkey (Huang *et al*., 2016). The hamster also exhibits similar CETP activity as man, which is absent in the rat (Huang *et al*., 2016).

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REFERENCES

Afonso, M S; Lavrador, M S F; Koike, M K; Cintra, D E; Ferreira, F D; Nunes, V S; Castilho, G; Gioielli, L A; Bombo, R P and Catanozi, S (2016). Dietary interesterified fat enriched with palmitic acid induces atherosclerosis by impairing macrophage cholesterol efflux and eliciting inflammation. *J. Nutr. Biochem.*, *32*: 91-100.

Alfieri, A; Imperlini, E; Nigro, E; Vitucci, D; Orrù, S; Daniele, A; Buono, P and Mancini, A (2018). Effects of plant oil interesterified triacylglycerols on lipemia and human health. *Int. J. Mol. Sci.*, *19(1)*: 104.

Ainie, K; Siew, W L; Tan, Y A; Nor Aini, I; Mohtar, Y; Tang, T S and Nuzul Amri, I (2004). *MPOB Test Methods – A Compendium of Test on Palm Oil Products, Palm Kernel Products, Fatty Acids, Food Related Products and Others*. MPOB, Bangi.

AOCS Official Method (1998). *Official Methods and Recommended Practices of the AOCS*. 5th edition. American Oil Chemists' Society. Champaign, IL USA. Ch 1-91.

Barlic, J; Zhu, W and Murphy, P M (2009). Atherogenic lipids induce high-density lipoprotein uptake and cholesterol efflux in human macrophages by up-regulating transmembrane chemokine CXCL16 without engaging CXCL16-dependent cell adhesion. *J. Immunol.*, *182(12)*: 7928-7936.

Berry, S E (2009). Triacylglycerol structure and interesterification of palmitic and stearic acid-rich fats: An overview and implications for cardiovascular disease. *Nutr. Res. Rev.*, *22(1)*: 3-17.

Borén, J and Williams, K J (2016). The central role of arterial retention of cholesterol-rich apolipoprotein-B-containing lipoproteins in the pathogenesis of atherosclerosis: A triumph of simplicity. *Curr. Opin. Lipidol.*, *27(5)*: 473-483.

Chen, J and Liu, H (2020). Nutritional indices for assessing fatty acids: A mini-review. *Int. J. Mol. Sci.*, *21(16)*: 5695.

Dorfman, S E; Wang, S; Vega-López, S; Jauhiainen, M and Lichtenstein, A H (2005). Dietary fatty acids and cholesterol differentially modulate HDL cholesterol metabolism in Golden-Syrian hamsters. *J. Nutr.*, *135(3)*: 492-498.

Escolà-Gil, J C; Llaverias, G; Julve, J; Jauhiainen, M; Méndez-González, J and Blanco-Vaca, F (2011). The cholesterol content of Western diets plays a major role in the paradoxical increase in high-density lipoprotein cholesterol and upregulates the macrophage reverse cholesterol transport pathway. *Arterioscler. Thromb. Vasc. Biol.*, *31(11)*: 2493-2499.

Ference, B A; Ginsberg, H N; Graham, I; Ray, K K; Packard, C J; Bruckert, E; Hegele, R A; Krauss, R M; Raal, F J; Schunkert, H; Watts, G F; Borén, J; Fazio, S; Horton, J D; Masana, L; Nicholls, S J; Nordestgaard, B G; Sluis, B; Taskinen, M R; Tokgözoğlu, L; Landmesser, U; Laufs, U; Wiklund, O; Stock, J K; Chapman, M J and Catapano, A L (2017). Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. *Eur. Heart J.*, *38(32)*: 2459-2472.

Filippou, A; Berry, S E; Baumgartner, S; Mensink, R P and Sanders, T A (2014). Palmitic acid in the sn-2 position decreases glucose-dependent insulinotropic polypeptide secretion in healthy adults. *Eur. J. Clin. Nutr.*, *68(5)*: 549-554.

Folch, J; Lees, M and Sloane Stanley, G H (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.*, *226(1)*: 497-509.

Fungwe, T V; Kudchodkar, B J; Lacko, A G and Dory, L (1998). Fatty acids modulate lecithin: Cholesterol acyltransferase secretion independently of effects on triglyceride secretion in primary rat hepatocytes. *J. Nutr.*, *128(8)*: 1270-1275.

Getz, G S and Reardon, C A (2017). Genetic control of apoprotein AI and atheroprotection: Some insights from inbred strains of mice. *Curr. Opin. Lipidol.*, *28(5)*: 403.

Goldstein, J L and Brown, M S (2009). The LDL receptor. *Arterioscler Thromb Vasc. Biol.*, *29*: 431-438.

Haerer, W; Delbaere, K; Bartlett, H; Lord, S and Rowland, J (2012). Relationships between HMG-CoA reductase inhibitors (statin) use and strength, balance and falls in older people. *Intern. Med. J., 42(12)*: 1329-1334.

Han, H; Xin, P; Zhao, L; Xu, J; Xia, Y; Yang, X; Sun, X and Hao, L (2012). Excess iodine and high-fat diet combination modulates lipid profile, thyroid hormone, and hepatic LDLr expression values in mice. *Biol. Trace Elem. Res., 147(1)*: 233-239.

Hatahet, W; Cole, L; Kudchodkar, B J and Fungwe, T V (2003). Dietary fats differentially modulate the expression of lecithin: Cholesterol acyltransferase, apoprotein-A1 and scavenger receptor b1 in rats. *J. Nutr., 133(3)*: 689-694.

Huang, H; Xie, Z; Yokoyama, W; Yu, L and Wang, T T Y (2016). Identification of liver CYP51 as a gene responsive to circulating cholesterol in a hamster model. *J. Nutr. Sci., 5*: E16.

Jo, H; Choe, S S; Shin, K C; Jang, H; Lee, J H; Seong, J K; Back, S H and Kim, J B (2013). Endoplasmic reticulum stress induces hepatic steatosis via increased expression of the hepatic very low-density lipoprotein receptor. *Hepatology, 57(4)*: 1366-1377.

Kris-Etherton, P M and Dietschy, J (1997). Design criteria for studies examining individual fatty acid effects on cardiovascular disease risk factors: Human and animal studies. *Am. J. Clin. Nutr., 65(5)*: 1590S-1596S.

Kritchevsky, D; Tepper, S A; Kuksis, A; Eghtedary, K and Klurfeld, D M (1998). Cholesterol vehicle in experimental atherosclerosis. 21. Native and randomized lard and tallow. *J. Nutr. Biochem., 9(10)*: 582-585.

Kritchevsky, D; Tepper, S A; Chen, S C; Meijer, G W and Krauss, R M (2000). Cholesterol vehicle in experimental atherosclerosis. 23. Effects of specific synthetic triglycerides. *Lipids, 35(6)*: 621-625.

Lavrador, M S F; Afonso, M S; Cintra, D E; Koike, M; Nunes, V S; Demasi, M; Lin, C J; Beda, L M M; Gioielli, L A; Bombo, R P A; Machado, R M; Catanozi, S; Nakandakare, E R and Lottenberg, A M (2019). Interesterified fats induce deleterious effects on adipose tissue and liver in LDLr-KO mice. *Nutrients, 11(2)*: 466.

Lee, M-N; Kim, J-R; Huh, H J; Lee, S-Y; Kang, E-S and Park, H-D (2014). Evaluation of the analytical performance of a direct quantitative assay of small dense LDL. *J. Lab. Med. Qual. Assur., 36(2)*: 84-91.

Lichtenstein, A H; Ausman, L M; Jalbert, S M and Schaefer, E J (1999). Effects of different forms of dietary hydrogenated fats on serum lipoprotein cholesterol levels. *N. Engl. J. Med., 340(25)*: 1933-1940.

Lida, H M D N; Sundram, K; Siew, W L; Aminah, A and Mamot, S (2002). TAG composition and solid fat content of palm oil, sunflower oil, and palm kernel olein belends before and after chemical interesterification. *J. Am. Oil Chem. Soc., 79(11)*: 1137-1144.

Marques, L R; Diniz, T A; Antunes, B M; Rossi, F E; Caperuto, E C; Lira, F S and Gonçalves, D C (2018). Reverse cholesterol transport: Molecular mechanisms and the non-medical approach to enhance HDL cholesterol. *Front. Physiol., 9*: 526.

Mensink, R P; Zock, P L; Kester, A D M and Katan, M B (2003). Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: A meta-analysis of 60 controlled trials. *Am. J. Clin. Nutr., 77(5)*: 1146-1155.

Mensink, R P; Sanders, T A; Baer, D J; Hayes, K C; Howles, P N and Marangoni, A (2016). The increasing use of interesterified lipids in the food supply and their effects on health parameters. *Adv. Nutr., 7(4)*: 719-729.

Nestel, P J; Noakes, M; Belling, G B; McArthur, R and Clifton, P M (1995). Effect on plasma lipids of interesterifying a mix of edible oils. *Am. J. Clin. Nutr., 62(5)*: 950-955.

Ng, Y T; Voon, P T; Ng, T K W; Lee, V K M; Mat Sahri, M; Mohd Esa, N; Ong, S H and Ong, A S H (2018). Interesterified palm olein (IEPalm) and interesterified stearic acid-rich fat blend (IEStear) have no adverse effects on insulin resistance: A randomized control trial. *Nutrients, 10(8)*: 1112.

Notarnicola, M; Messa, C; Refolo, M G; Tutino, V; Miccolis, A and Caruso, M G (2010). Synergic effect of eicosapentaenoic acid and lovastatin on gene expression of HMGCoA reductase and LDL receptor in cultured HepG2 cells. *Lipids Health Dis., 9(1)*: 1-8.

O'Reilly, M; Dillon, E; Guo, W; Finucane, O; Mcmorrow, A; Murphy, A; Lyons, C; Jones, D; Ryan, M; Gibney, M; Gibney, E; Brennan, L; Moya, M L; Reilly, M P; Roche, H M and McGillicuddy, F C (2016). High-density lipoprotein proteomic composition, and not efflux capacity, reflects differential modulation of reverse cholesterol

transport by saturated and monounsaturated fat diets. *Circulation*, *133(19)*: 1838-1850.

Oshio, Y; Hattori, Y; Kamata, H; Ozaki-Masuzawa, Y; Seki, A; Tsuruta, Y and Takenaka, A (2021). Very low-density lipoprotein receptor increases in a liver-specific manner due to protein deficiency but does not affect fatty liver in mice. *Sci. Rep.*, *11(1)*: 8003.

Parveez, G K A; Hishamuddin, E; Loh, S K; Ong-Abdullah, M; Salleh, K M; Bidin, M N I Z; Sundram, S; Hasan, Z A A and Idris, Z (2020). Oil palm economic performance in Malaysia and R&D progress in 2019. *J. Oil Palm Res.*, *32(2)*: 159-190.

Plump, A S; Scott, C J and Breslow, J L (1994). Human apolipoprotein AI gene expression increases high density lipoprotein and suppresses atherosclerosis in the apolipoprotein E-deficient mouse. *Proc. Natl. Acad. Sci.*, *91(20)*: 9607-9611.

Reena, M B and Lokesh, B R (2007). Hypolipidemic effect of oils with balanced amounts of fatty acids obtained by blending and interesterification of coconut oil with rice bran oil or sesame oil. *J. Agric. Food Chem.*, *55(25)*: 10461-10469.

Reena, M B; Gowda, L R and Lokesh, B R (2011). Enhanced hypocholesterolemic effects of interesterified oils are mediated by upregulating LDL receptor and cholesterol 7-α-hydroxylase gene expression in rats. *J. Nutr.*, *141(1)*: 24-30.

Robinson, D M; Martin, N C; Robinson, L E; Ahmadi, L; Marangoni, A G and Wright, A J (2009). Influence of interesterification of a stearic acid-rich spreadable fat on acute metabolic risk factors. *Lipids*, *44(1)*: 17-26.

Rudling, M; Angelin, B; Ståhle, L; Reihnér, E; Sahlin, S; Olivecrona, H; BjÖrkhem, I and Einarsson, C (2002). Regulation of hepatic low-density lipoprotein receptor, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and cholesterol 7α-hydroxylase mRNAs in human liver. *J. Clin. Endocrinol. Metab.*, *87(9)*: 4307-4313.

Sacks, F M; Lichtenstein, A H; Wu, J H Y; Appel, L J; Creager, M A; Kris-Etherton, P M; Miller, M; Rimm, E B; Rudel, L L; Robinson, J G; Stone, N J and Horn, L V V (2017). Dietary fats and cardiovascular disease: A presidential advisory from the American Heart Association. *Circulation*, *136(3)*: e1-e23.

Sanders, T A B; Filippou, A; Berry, S E; Baumgartner, S and Mensink, R P (2011). Palmitic acid in the *sn*-2 position of triacylglycerols acutely influences postprandial lipid metabolism. *Am. J. Clin. Nutr.*, *94(6)*: 1433-1441.

Shen, W J; Asthana, S; Kraemer, F B and Azhar, S (2018). Scavenger receptor B type 1: Expression, molecular regulation, and cholesterol transport function. *J. Lipid Res.*, *59(7)*: 1114-1131.

Spady, D K; Kearney, D M and Hobbs, H H (1999). Polyunsaturated fatty acids up-regulate hepatic scavenger receptor B1 (SR-BI) expression and HDL cholesteryl ester uptake in the hamster. *J. Lipid Res.*, *40(8)*: 1384-1394.

Sundram, K; Karupaiah, T and Hayes, K C (2007). Stearic acid-rich interesterified fat and *trans*-rich fat raise the LDL/HDL ratio and plasma glucose relative to palm olein in humans. *Nutr. Metab.*, *4(1)*: 3.

Tarmizi, A H A; Lin, S W and Kuntom, A (2008a). Palm-based standard reference materials for iodine value and slip melting point. *Anal. Chem. Insights*, *3*: 127-133.

Tarmizi, A H A; Siew, W L and Kuntom, A (2008b). Production of palm oil reference materials for the determination of solid fat content. *J. Food Qual.*, *31(6)*: 673-685.

Tiebel, O; Oka, K; Robinson, K; Sullivan, M; Martinez, J; Nakamuta, M; Ishimura-Oka, K and Chan, L (1999). Mouse very low-density lipoprotein receptor (VLDLR): Gene structure, tissue-specific expression and dietary and developmental regulation. *Atherosclerosis*, *145(2)*: 239-251.

Ulbricht, T L V and Southgate, D A T (1991). Coronary heart disease: Seven dietary factors. *Lancet, 338(8773)*: 985-992.

Yli-Jokipii, K; Kallio, H; Schwab, U; Mykkänen, H; Kurvinen, J P; Savolainen, M J and Tahvonen, R (2001). Effects of palm oil and transesterified palm oil on chylomicron and VLDL triacylglycerol structures and postprandial lipid response. *J. Lipid Res.*, *42(10)*: 1618-1625.

Zelcer, N and Tontonoz, P (2006). Liver X receptors as integrators of metabolic and inflammatory signaling. *J. Clin.I Invest.*, *116(3)*: 607-614.

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PALM GAMMA-TOCOTRIENOL SUPPLEMENTATION SUPPRESS TUMOUR GROWTH AND METASTASIS IN A SYNGENEIC MOUSE MODEL OF BREAST CANCER

SHONIA SUBRAMANIAM[1,2]; AMMU KUTTY RADHAKRISHNAN[3]; JEYA SEELA ANANDHA RAO[4]; PREMDASS RAMDAS[5]; NG MEI HAN[2]; METHIL KANNAN KUTTY[6] and KANGA RANI SELVADURAY[2]*

ABSTRACT

Gamma-tocotrienol (γT3) is an isoform of vitamin E found abundantly in palm oil, which is reported to possess antioxidant and anticancer activities. However, the immune-modulating properties of γT3 have yet to be elucidated. Breast cancer (BC) was induced in female BALB/c mice by injecting 4T1 murine mammary cancer cells into their mammary fat pads. When the tumour was palpable, the animals were randomly assigned into two groups: (i) control [fed twice daily with 50 µL vehicle (soy oil)] or (ii) experimental (fed twice daily with 50 µL of 0.5 mg of γT3). Results show that mice fed with γT3 had reduced tumour growth and metastasis. However, there are no marked changes in the percentages of peripheral blood leukocytes and cytokines production in these animals. Immunohistochemistry using antibodies to murine CD4, IL12Rβ2, IL24 and FoxP3 on tumour sections from γT3 treated mice suggested that γT3 induced suppression of tumour growth and metastasis as well as reduced immunosuppression in the tumour microenvironment. It can be concluded that, γT3 has the potential to suppress tumour growth and metastasis in this model. Further investigation on the host immune response is possible by prolonging treatment duration against BC.

Keywords: breast cancer, gamma-tocotrienol, mouse, supplementation, 4T1.

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INTRODUCTION

Breast cancer (BC) is ranked as the most frequent cancer among women worldwide; with an estimated 11.6% new cases as reported in the global cancer statistics 2018 (Bray *et al.*, 2018). According to the Malaysian National Cancer Registry (2012-2016) report (Azizah *et al.*, 2019), one in 20 women may develop BC in their lifetime and the incidence of BC was highest in Chinese, followed by Indians and Malays (Azizah *et al.*, 2019). The mean age standardised rate (ASR) amongst Malaysian women who developed BC was reported to be 39.3 per 100 000 populations in 2006 (Azizah *et al.*, 2019). The survival rate of patients with BC is considered crucial as it can control the mortality rate; and in Malaysia, the 5-year survival rate was found to be favourably improved (Abdullah *et al.*, 2013). Use of alternative medicine, particularly dietary supplements were reported to be common practices among Malaysian BC survivors (Shaharudin *et al.*, 2011).

Bioactive compounds derived from natural sources are preferred compared to synthetic medicinal products as these have lesser side-effects. One such compound is tocotrienols (T3),

[1] Faculty School of Postgraduate Studies, International Medical University, 126, Jalan Jalil Perkasa 19, Bukit Jalil, 57000 Kuala Lumpur, Malaysia.

[2] Malaysian Palm Oil Board, 6 Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang, Selangor, Malaysia.

[3] Jeffery Cheah School of Medicine, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, 47500 Subang Jaya, Selangor, Malaysia.

[4] Pathology Division, School of Medicine, International Medical University, 126, Jalan Jalil Perkasa 19, Bukit Jalil, 57000 Kuala Lumpur, Malaysia.

[5] Applied Biomedical Sciences and Biotechnology, School of Health Sciences, International Medical University, 126, Jalan Jalil Perkasa 19, Bukit Jalil, 57000 Kuala Lumpur, Malaysia.

[6] Faculty of Medicine, Lincoln University, 2, Jalan Stadium SS 7/15, SS 7, 47301 Petaling Jaya, Selangor, Malaysia.

* *Corresponding author e-mail: krani@mpob.gov.my*

a natural isoform of vitamin E, which is found abundantly in palm oil and widely known for its anticancer, antioxidant and other beneficial health effects (Kabir *et al.*, 2017; Montagnani Marelli *et al.*, 2019). Vitamin E is divided into two families, namely tocopherol and T3. Major sources of dietary tocopherols are wheat-germ oil, safflower-seed oil, maize oil, soybean oil, whilst T3 can be found abundantly in palm oil, rice-bran oil and palm kernel oil. Both tocopherols and T3 exist naturally as four analogues *i.e.*, alpha (α), beta (β), delta (δ) and gamma (γ). Vitamin E from palm oil is known as tocotrienol-rich fraction (TRF), which contains all four T3 analogues and alpha-tocopherol (Mba *et al.*, 2015). There is increasing evidence suggesting T3s are potent antioxidants, which can potentially protect against various diseases, including cancer (Abraham *et al.*, 2019; Montagnani Marelli *et al.*, 2019). Evidence from cell-based studies have shown that T3 can inhibit growth and proliferation of various cancer cell lines (Aggarwal *et al.*, 2019; De Silva *et al.*, 2016; Fontana *et al.*, 2020; Ghanem *et al.*, 2019; Nesaretnam *et al.*, 1998). The ability of T3 to selectively inhibit the proliferation of cancer cells strongly support their anticancer potential. Furthermore, T3 is also credited for its immune-modulating activities in a syngeneic mouse model of BC. For instance, TRF supplementation was reported to induce cancer-specific immune responses in a syngeneic mouse model of BC (Hafid *et al.*, 2010). Previous studies had shown that T3 can inhibit both the proliferation of 4T1 mouse mammary cancer cells and tumour growth (Selvaduray *et al.*, 2010; Subramaiam *et al.*, 2021).

The focus of this article is to investigate the effects of daily supplementation of gamma-tocotrienol (γT3) to inhibit tumour growth and exert immunomodulation in a syngeneic mouse model of BC. The findings from this preclinical study may be useful to predict and develop new treatment strategies using palm T3 to fight BC.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The 4T1 mouse mammary cancer cells were obtained from the American Tissue Culture Collection (ATCC) (ATCC, Rockville, USA) and cultured according to the protocol provided by the ATCC. The cells were cultured at 37ºC in Roswell Park Memorial Institute Medium (RPMI) medium (GIBCO, Invitrogen, USA) supplemented with L-glutamine (GIBCO, Invitrogen, USA), 10% fetal bovine serum (FBS) (GIBCO, Invitrogen, USA), and 1% penicillin-streptomycin (GIBCO, Invitrogen, USA) in a humidified incubator containing 5% carbon dioxide (CO_2).

Preparation of Treatment

The γT3 used in this study was obtained from the Malaysian Palm Oil Board (MPOB). The γT3 was extracted from palm oil with a purity range of 95%-99% (Maarasyid *et al.*, 2014). The γT3 was prepared fresh every day prior to feeding by dissolving 0.5 mg of γT3 in 50 µL of soy oil, which served as the vehicle.

Experimental Procedure

Female BALB/c mice aged 5-6 weeks (Cheneur Suppliers, Malaysia) were housed at the animal holding facility (AHF), International Medical University (IMU). All experimental procedures were approved by the Joint Committee for Research and Ethics, IMU (IMU-R060-2010) and the study was conducted according to the IMU animal ethics guidelines. The mice were maintained at 25 ± 2°C with 12:12 hr dark/light cycle and they had free access to commercial food pellets and water throughout the study period. After one-week of acclimatisation, six (n=6) mice were sacrificed to collect baseline data. The remaining mice (n=48) received an injection of 100 µL of 4T1 cells (10 000 cells/mL) at their right thoracic mammary fat pad to induce BC (Selvaduray *et al.*, 2010). Once tumour was palpable (day 14), the mice were randomly divided into vehicle and treatment groups. The animals in the vehicle group were fed with 50 µL vehicle (soy oil) whilst the mice in the treatment group were fed with 50 µL of vehicle containing 0.5 mg γT3 twice daily (morning and evening) for a period of 35 days by oral gavage. Throughout the study period, six mice (n=6) from each group were sacrificed every seven days (day 14, 21, 28, 35). Tumour size was monitored and measured weekly using a digital calliper. Tumour volume was calculated using the formula: $V = 0.52 \times L^2 \times W$ (V: volume, L: length and W: width) (Selvaduray *et al.*, 2010). The study was terminated at day 35 when the tumour load in the vehicle group became too big and the animals started to show signs of distress. The animals were humanely euthanised using the cervical dislocation method.

Histopathology Assessment

At autopsy, tumour, lung, kidney and liver were removed and fixed in 10% formalin for 48 hr before it was processed using an automatic tissue processor (Leica TP1020 Automatic Tissue Processor, Leica, Germany). These tissues were paraffin-embedded and sectioned at 4 µm thickness using a rotary microtome (Rotary Microtome, Leica, Germany). The tissue sections were stained with haematoxylin and eosin (H&E) stains (Leica, Germany). The stained slides were evaluated by a pathologist using a light microscope (Nikon eclipse, Japan) to look for extent and signs of metastasis.

Immunohistochemistry Staining

Tumours sections taken from vehicle-fed and γT3-fed mice were incubated at room temperature with respective primary antibodies for 1 hr. The primary antibodies used were (i) anti-CD4 [rabbit anti-mouse CD4 polyclonal antibody (Dako, Denmark); (ii) anti-Forkhead box P3 (FoxP3) [rabbit anti-mouse FoxP3 polyclonal antibody] (Dako, Denmark); (iii) anti-interleukin-24 (IL-24) [rabbit anti-mouse IL-24 polyclonal antibody] (Dako, Denmark) and (iv) anti-interleukin-12-beta-2 receptor (IL-12Rβ2) [rabbit anti-mouse IL-12Rβ2 polyclonal antibody] (Dako, Denmark). After 1 hr, the slides were washed with a wash buffer and incubated for 20 min at room temperature with a biotinylated secondary antibody [biotinylated anti-rabbit polyclonal antibody] (Dako, Denmark)]. Following this, the slides were washed and incubated at room temperature for 20 min with streptavidin-conjugated horseradish peroxidase (HRP) (Dako, Denmark). After the slides were washed, a substrate chromogen (Dako, Denmark) was added to the sections and the slides were incubated at room temperature for an additional 20 min. Following this, the slides were counter-stained with haematoxylin and viewed under a light microscope. Staining intensity was determined using a semi-quantitative method of Allred scoring system (Fedchenko and Reifenrath, 2014), whereby the percentage of stained cells and intensity of staining were calculated. Each section was given a score based on the staining pattern (0= negative, 1= weak, 2= moderate and 3= strong).

Leucocyte Subsets

At autopsy, blood was obtained via cardiac puncture and collected into heparinised tubes. The blood was centrifuged (200 g for 10 min at 4°C) to separate plasma and cells. Once the plasma was removed, the red blood cells (RBC) were lysed by adding 2 mL of RBC lysis buffer (BD Pharm Lyse, USA) and the leucocytes were recovered by centrifugation (200 g for 5 min at 4°C). Then, the leucocytes were washed with cold phosphate-buffered saline (PBS) and recovered by centrifugation. Following this, the cells were resuspended in a staining buffer (FACS Sheath Fluid BD, USA) and counted. Cell count was adjusted to 1×10^6 / 100 µL cells. The cells were added to appropriately labelled tubes and stained with conjugated antibodies for flow cytometer analysis. The antibodies used were specific to murine (i) TCR-β^+CD4$^+$CD8A$^+$ to identify T-lymphocytes (Biosciences BD, USA); (ii) CD335$^+$CD3e$^-$CD49b$^+$ to detect natural killer (NK) cells (Biosciences BD, USA); and (iii) CD4$^+$CD25$^+$FR-4$^+$ to identify T-regulatory (Treg) cells (Biosciences BD, USA). The cells were stained for 20 min at room temperature in the dark. Following this, the cells were washed with a staining buffer and recovered by centrifugation. The stained cells were resuspended in 0.5 mL of cell fix buffer (Cell Fix BD, USA) and analysed using a multicolour flow cytometer (FACS Calibur, Becton-Dickson, USA). Data was collected using the Cell Quest software provided by the manufacturer (Becton Dickinson, USA). In each acquisition, 10 000 cells were collected for data analysis.

Quantification of Cytokines

When the animals were sacrificed, the spleen from each animal was aseptically removed and a splenocyte suspension was prepared. The cells were counted and plated in a 96-well plate at a cell density of 5×10^3 splenocytes / well. The plates were incubated at 37°C in a humidified incubator with 5% CO_2. Then, 100 µL of 4T1 murine mammary cancer cells that have been pre-treated with 25 µg mL^{-1} mitomycin C (MMC) (Sigma-Aldrich, USA) for 2 hr were added to the wells containing the freshly plated splenocytes. The plate was incubated at 37°C for 72 hr in a humidified 5% CO_2 incubator. After 72 hr, the splenocytes were harvested into 1.5 mL tubes and centrifuged (200 g for 10 min at 4°C). The culture supernatant from each sample was collected and stored at -80°C prior to quantification of interferon gamma (IFN-γ) and transforming growth factor β (TGF-β) using commercial READY SET GO! enzyme-linked immunosorbent assay (ELISA) kits as recommended by the manufacturer (eBiosciences, San Diego, Inc.).

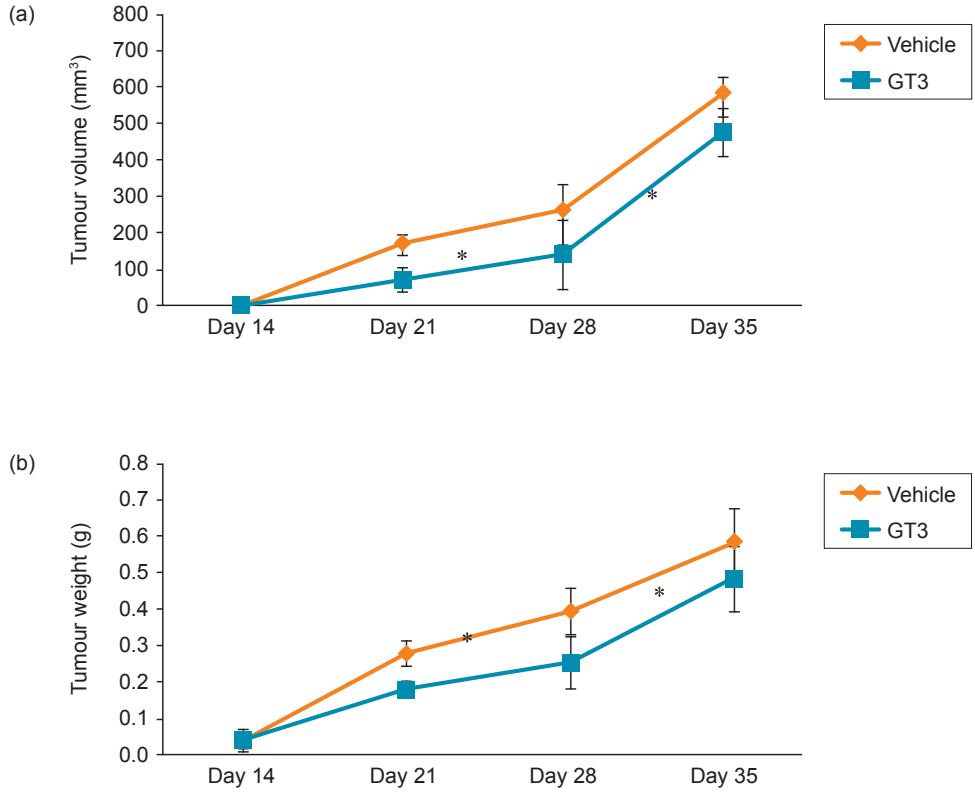
Statistical Analysis

One-way analysis of variance (ANOVA) was performed using Statistical Package for Social Science (SPSS) version 16. The *post-hoc* Dunnett's test was used to compare data of experimental group against control groups. All data points are expressed as the mean ± standard deviation (SD). A p-value of less than 0.05 ($p<0.05$) was considered to be statistically significant.

RESULTS AND DISCUSSION

There was a significant delay in tumour progression on days 21 and 28 in mice fed with γT3 compared to the vehicle-fed animals as shown by the tumour volume (*Figure 1a*) and tumour weight (*Figure 1b*) measurements. Histopathology findings on tumour sections from γT3-fed mice exhibited pleomorphic features with hyperchromatic nucleus

and the presence of necrosis (*Figure 2*) whilst the breast tissues sections from the vehicle-fed mice showed poorly differentiated tumour and marked metastasis. In addition, sections obtained from the vital organs of animals fed with γT3, such as the lung (*Figure 3a*), liver (*Figure 3b*) and kidney (*Figure 3c*) showed signs of minimal or delayed metastasis when compared to the vehicle-fed group. The lung tissue sections from both groups showed clusters of malignant (yellow circle) inflammatory (indicated with white circle) cells (*Figure 3a*). Sections of liver tissues from both groups also showed similar features (*Figure 3b*). However, sections of kidney tissue from both groups showed normal architecture with well-preserved glomerular and tubular structures (red circle) (*Figure 3c*). However, the tissue sections from the liver and lungs from the vehicle-fed mice showed presence of multiple metastasis foci, which appear to have developed rapidly (*Table 1*). These findings suggest that γT3 supplementation inhibited development and progression of BC as well as blocking the onset of metastasis. Previous studies have found that daily supplementation with TRF inhibited tumour growth and metastasis in this highly invasive syngeneic mouse model of BC (Abdul Hafid *et al.*, 2013). Metastasis of tumour to distant organs has been associated with higher mortality (Ording *et al.*, 2017) and in BC patients, tumour usually metastasises to their lungs (Medeiros and Allan, 2019) causing further complication. Inhibition of tumour growth following γT3 supplementation observed in this study is in agreement with our previous studies, where we had reported that daily supplementation of TRF inhibited tumour growth and metastasis in this syngeneic murine model of BC (Abdul Hafid *et al.*, 2013; Abdul Hafid and Radhakrishnan, 2019; Selvaduray *et al.*, 2010; Weng-Yew *et al.*, 2009). Besides these evidence, recent studies also showed that γT3 significantly suppressed proliferation and invasion of prostate (Fontana *et al.*, 2020), gastrointestinal (Zhang *et al.*, 2018), colon (Aggarwal *et al.*, 2019; Wada *et al.*, 2017) and liver (Aggarwal *et al.*, 2019; Sazli *et al.*, 2015) cancer cells. The underlying mechanism of how γT3 suppresses tumour growth could be due to its anti-angiogenesis (Selvaduray *et al.*, 2012), anti-proliferative (Nesaretnam *et al.*, 1995) and anti-apoptotic (Srivastava and Gupta, 2006; Wu and Ng, 2010) properties; which are well-documented in the literature. However, in the present study, we intent to investigate the involvement of CD4, FoxP3, IL-24, IL-12Rβ2 in the γT3 mediated suppression of tumour growth and metastases.



Note: Tumour volume and weight were measured once every seven days (days 14, 21, 28 and 35) using a digital calliper. Tumour volume (mm^3) was calculated using a formula that was previously described (Selvaduray *et al.*, 2010). Tumour weight was measured by weighing the tumour at autopsy. Each data point represents mean (n=6) ± SD.

Figure 1. (a) Tumour volume, and (b) tumour weight.

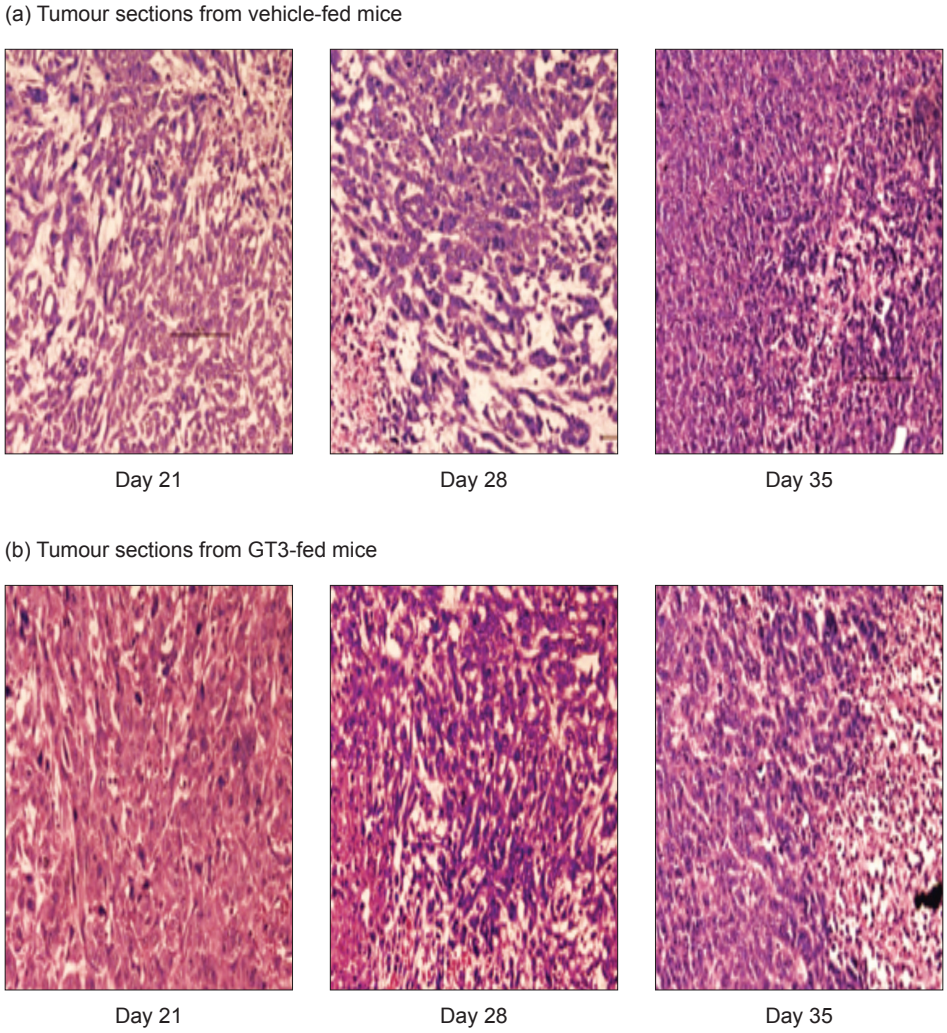
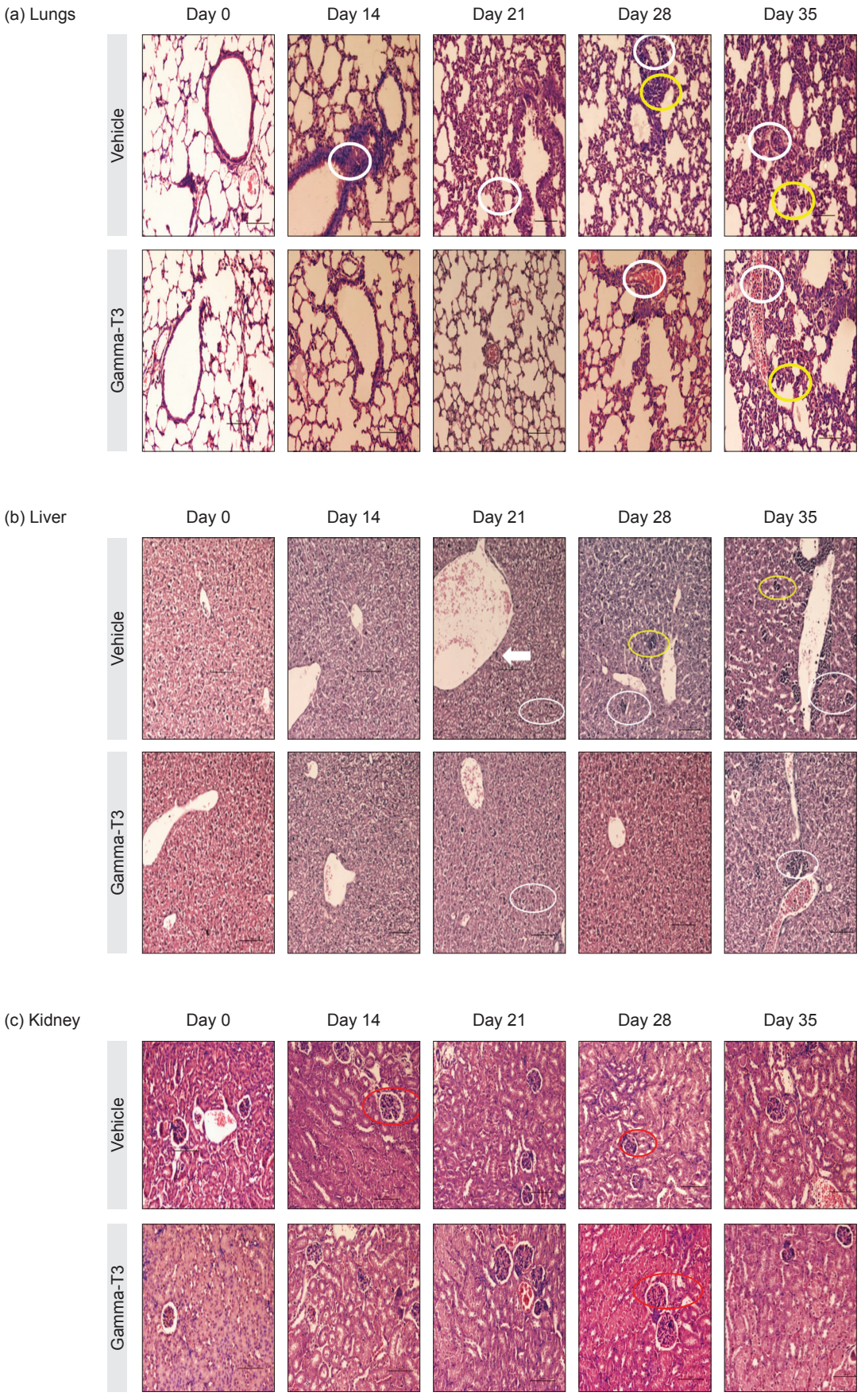


Figure 2. Photomicrograph images (200X) of H&E stained sections of breast tumour tissues collected on day 21, 28 and 35 from animals fed with vehicle or γT3 (GT3) groups.

TABLE 1. KRUSKAL-WALLIS TEST OF LUNG AND LIVER METASTASIS

Groups	Number of mouse	Breast tumour differentiation	Metastasis	
			Lung	Liver
Vehicle	1	Poorly differentiated	+	+
	2	Poorly differentiated	+	+
	3	Poorly differentiated	+	+
	4	Poorly differentiated	+	+
	5	Moderately differentiated	-	-
	6	Moderately differentiated	-	-
γT3	1	Necrosis	-	-
	2	Moderately differentiated	+	-
	3	Necrosis	-	-
	4	Necrosis	-	-
	5	Necrosis	-	-
	6	Poorly differentiated	+	+

Note: The metastasis deposits in the lung and liver of vehicle and γT3 groups were compared as present (+) and absent (-). (p<0.05) for lung and liver.



Note: Malignant cells indicated with yellow circle; inflammatory cells indicated with white circle.

Figure 3. Photomicrograph images (200X) of H&E stained (a) lungs; (b) liver and (c) kidney sections collected at autopsy on day 0, 14, 21, 28 and 35 days following tumour induction in mice fed with from vehicle or γT3 (GT3).

CD4 is a cell surface glycoprotein found on surface of T-helper (Th) and Treg cells (Sambucci *et al*., 2019), which has high specificity to major histocompatibility complex class II (MHC II) proteins (Rzepecka *et al*., 2019). Activation of $CD4^+$ T-cells can result in differentiation of sub-types that facilitate immune regulation through secretion of certain cytokines. A previous study by Huang *et al*. (2015) suggested that $CD4^+$, along with $CD8^+$ T-cells are dynamically involved in the immune response against BC, and that Th1 and $CD8^+$ T-cells were dominant populations in tumour-infiltrating lymphocytes involved in the immunosurveillance in the early stage of BC development. However, in the late cancer stage, the number of $CD4^+$ tumour-infiltrating lymphocytes increased significantly and the Treg and Th17 cells become the dominant populations, which contribute to the tumour promotion. The high number of $CD4^+$ T-cells are most likely Treg cells, which also express this cell surface protein. High numbers of Treg cells are indicative of poor prognosis as these cells will create an immunosuppressive condition in the tumour microenvironment (Knochelmann *et al*., 2018; Ohue and Nishikawa, 2019). Hence, tumour will be able to progress due to the compromised cell-mediated immunity and reduction in the T-cell populations. BC patients and experimental animals induced with BC with advanced cancer frequently report dysfunctions in the immune system, which is evident from the reduced $CD4^+$ to $CD8^+$ ratio ($CD4^+$:$CD8^+$) and decreased T-cell proliferation possibly due to suppression by the Treg cells. In the present study, tumour tissue sections from γT3-fed mice showed higher number of $CD4^+$ cells compared to tumour sections from vehicle-fed animals (*Figure 4*), suggestive of $CD4^+$ associated anti-tumour immunity induced by γT3. However, it should be noted that Treg cells also express the CD4 glycoprotein on the cell surface (Yu *et al*., 2012).

The transcription factor FoxP3 is a key biomarker that can be used to identify Treg cells (Tanaka and Sakaguchi, 2019). Tumour tissue sections from vehicle fed mice showed presence of high number of $FoxP3^+$ cells (*Figure 4*). High number of $FoxP3^+$ cells in tumour microenvironment is associated with suppression of host immune response, which in turn can allow tumour progression to take place (Sasidharan and Elkord, 2018; Verma *et al*., 2019). However, tumour sections from γT3-fed mice showed marked reduction in the number of $FoxP3^+$ cells (*Figure 4*). These findings suggest that γT3 supplementation can facilitate the host immune system to suppress tumour progression by limiting the immunosuppressive tumour microenvironments.

Tumour sections from γT3-fed animals showed higher expression of IL-24 and IL-12Rβ2 compared to sections from vehicle-fed mice (*Figure 4*), which supported presence of cell-mediated immunity. Interleukin-24 is cytokine linked with inhibition of tumour growth, anti-angiogenic and anti-metastasis activities (Panneerselvam *et al*., 2019; Zhang *et al*., 2019) whereby IL-12Rβ2 together with IFN-γ mediates differentiation of Th1 subset that plays crucial roles in cell-mediated immune responses, which include anticancer effects (Yamamoto *et al*., 1997). Statistical analysis using a semi-quantitative method confirmed that the difference observed on the expression of the four biomarkers (CD4, IL-24, IL-12β2R and FoxP3) in tumour tissues section from vehicle- or γT3-fed mice were statistically significant (p<0.05) (*Figure 5*).

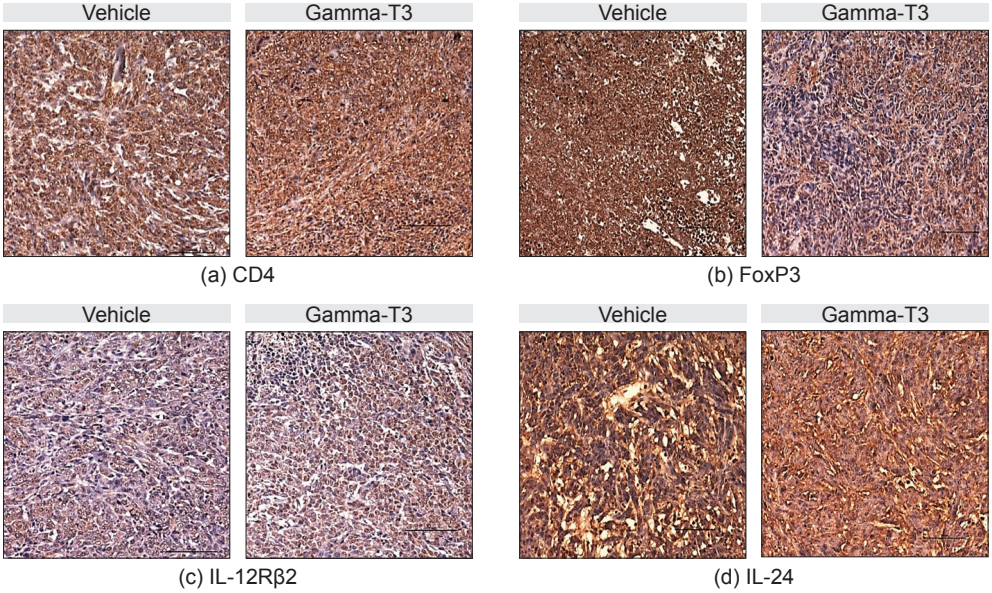
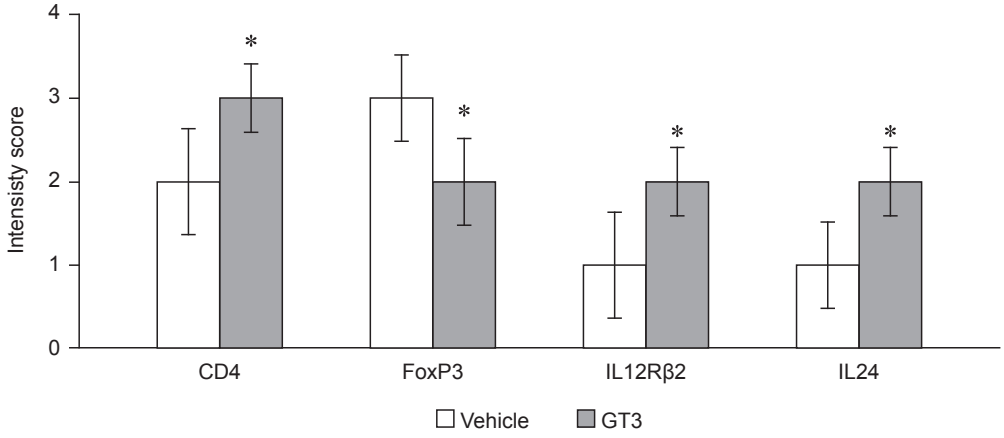


Figure 4. Photomicrograph images (200X) of breast tumour tissue sections from vehicle or γT3-fed mice stained with antibodies to murine (a) CD4, (b) FoxP3, (c) IL-12Rβ2 and (d) IL-24 biomarkers analysed using immunohistochemistry.



Note: This scoring system includes staining intensity and percentage of cells expressing these biomarkers. The results are expressed with an IHC score of (0= negative, 1= weak, 2= moderate and 3= strong). Results expressed as a percentage ± SD of six mice per group and * $p<0.05$ against vehicle group.

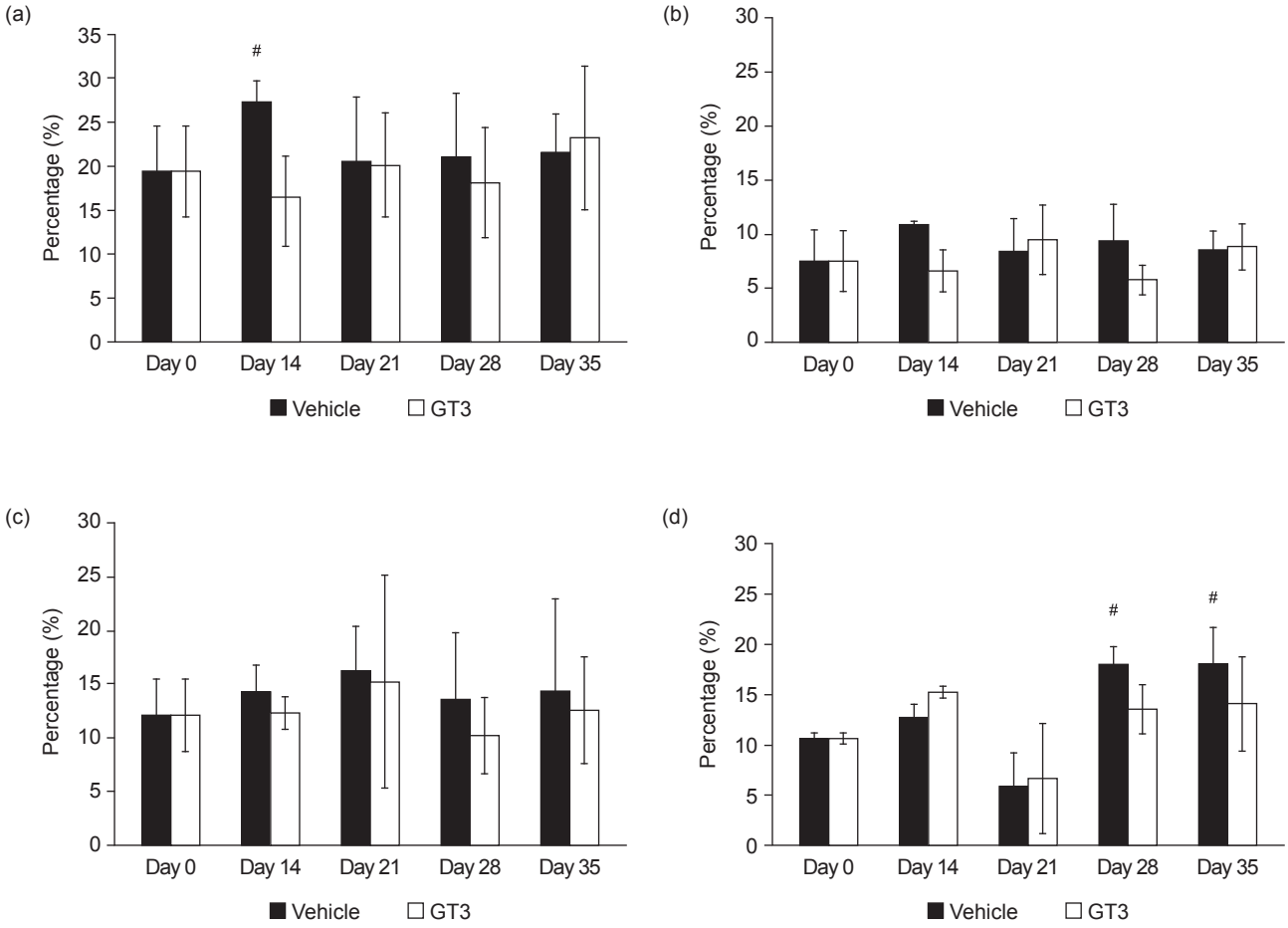
Figure 5. Immunohistochemistry staining of the four antibodies CD4, FoxP3, IL-12Rβ2 and IL-24 on the tumour sections from vehicle or γT3 (GT3) - fed mice were scored using a semi-quantitative method i.e.*, Allred Scoring System.*

There was no significant difference ($p>0.05$) in the percentage of T-lymphocytes that express $CD4^+$ (TH) or $CD8^+$ (cytotoxic T-lymphocytes) as well as NK cells in peripheral blood at any of the time-points studied (*Figure 6*). There was a significant ($p<0.05$) increase in the Treg cell population in peripheral blood from vehicle-fed mice on day 28 and day 35 (*Figure 6*). The increase in the number of Tregs appears to correlate with bigger tumour load (*Figure 1*). These findings support the results from the immunohistochemistry analysis, which showed reduction in $FoxP3^+$ cells in the tumour tissue sections from γT3 fed mice. This provided more evidence to support that γT3 supplementation can modulate the host immune system in this syngeneic mouse model of BC.

However, it should be noted that γT3 supplementation did not completely stop tumour progression. One of the reasons for this observation may be because γT3 supplementation only started once the tumour is palpable. The syngeneic mouse model of BC used in this study is a highly tumourigenic model (Pulaski and Ostrand-Rosenberg, 2000). Hence, intervention with γT3 once tumour cells have been inoculated may give better results as this could trigger early activation of the host immune system to fight cancer. Another reason could be the low bioavailability of γT3 in the blood as previous studies have found that oral γT3 has low bioavailability due to low intestinal permeability (Abuasal *et al.*, 2012).

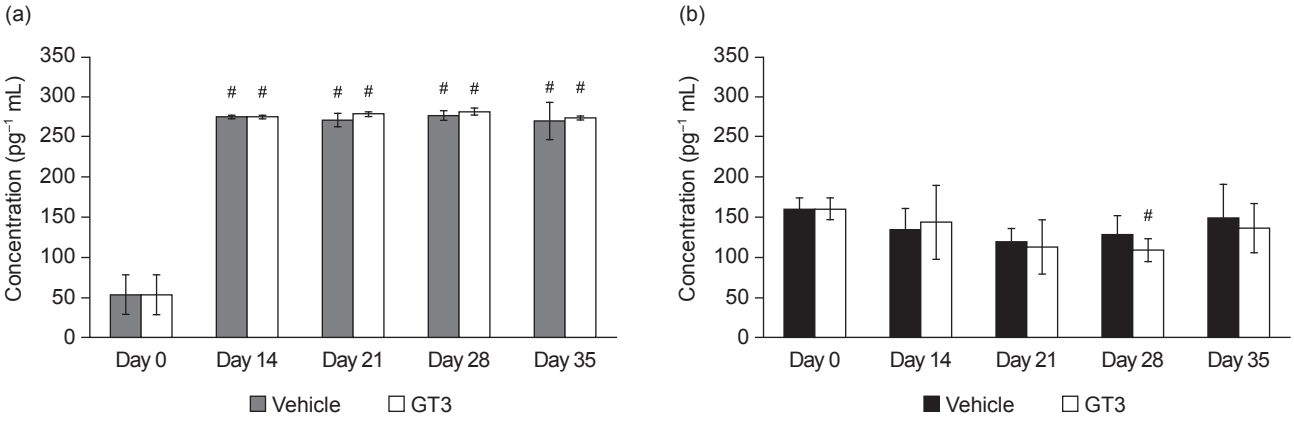
Culture supernatant from mitogen-stimulated splenocytes from vehicle- and γT3-fed mice showed significantly higher levels of IFN-γ (*Figure 7*) when compared to baseline (day 0). However, there was no significant difference between the two study groups. IFN-γ is the main regulator for TH1 immune response, which has a crucial role in controlling tumour growth (Alizadeh *et al.*, 2021) whilst TGF-β is a major cytokine that supports activities of Treg cells (Mikami *et al.*, 2020), which play an important role in immunosuppression *i.e.*, allow tumour progression. The lack of significant difference in the production of these cytokines between the two study groups may be attributed to the delay in starting the γT3 intervention in this model. It is also possible that feeding γT3 for a short duration may not be sufficient to activate host immune response to secrete sufficient cytokines to suppress the cancer. The syngeneic mouse model of BC used in the present study is a highly invasive and spontaneously metastatic model. Hence, short term feeding of γT3 once tumour is palpable, may be insufficient to prepare the host immune system to control the tumour. Previous studies that have looked at effects of TRF supplementation in tumour-induced mice vaccinated with dendritic cell (DC) vaccines before (Abdul Hafid and Radhakrishnan, 2019; Abdul Hafid *et al.*, 2013; Hafid *et al.*, 2010) showed enhanced immune function and tumour suppression. However, it should be noted that in both these studies, the animals were supplemented with TRF for a longer period, which may have been sufficient to regulate the host immune system to fight against cancer.

To date, there has been only one study that investigated the effects of using TRF from palm oil in combination with tamoxifen in human BC patients (Nesaretnam *et al.*, 2010). This study reported no association between adjuvant tocotrienol therapy and BC-specific survival in women with early BC, which has posed a setback to carry out more robust clinical trials in BC patients. More studies are needed to further evaluate the potential of using TRF as an anticancer agent in BC patients.



Note: Leucocytes were isolated from peripheral blood (vehicle or γT3 (GT3)-fed mice) by via cardiac puncture and stained with fluorochrome-labelled antibodies to various murine cell surface proteins and analysed using a flow-cytometer. Data was collected using the Cell Quest software provided by the manufacturer (Becton Dickinson, USA). In each acquisition, 10 000 cells were collected for data analysis. Results expressed as mean percentage ± SD of 6 mice per group. # $p<0.05$ when each group is compared against baseline (day 0); * $p<0.05$ when γT3 group is compared against vehicle group.

Figure 6. Percentage of (a) $CD4^+$ T-cells, (b) $CD8^+$ T-cells, (c) NK cells, and (d) Treg cell in peripheral blood analysed using a flow cytometer.



Note: Culture supernatant was harvested after 72 hr and analysed for (a) IFN-g and (b) TGF-β using commercial ELISA kits. Results expressed as mean percentage ± SD of 6 mice per group. # Significantly different ($p<0.05$) from baseline (day 0).

Figure 7. Splenocytes obtained from spleens of mice fed with vehicle or γT3 (GT3)-fed mice cultured in the presence of mitomycin-C treated 4T1 cells.

CONCLUSION

This study shows that supplementing mice with 0.5 mg twice daily with γT3 showed promising results with regards to its anticancer effects based on the tumour growth and histopathology results. These findings suggest that γT3 provided via the oral route has reached the breast tumour tissues and was able to exert some anticancer effects in this mouse model. However, γT3 supplementation did not appear to have a significant effect on modulation of host immune system in this model.

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REFERENCES

Abdul Hafid, S R and Radhakrishnan, A K (2019). Palm tocotrienol-adjuvanted dendritic cells decrease expression of the SATB1 gene in murine breast cancer cells and tissues. *Vaccines*, *7(4)*: 198. DOI: 10.3390/vaccines7040198.

Abdul Hafid, S R; Chakravarthi, S; Nesaretnam, K and Radhakrishnan, A K (2013). Tocotrienol-adjuvanted dendritic cells inhibit tumor growth and metastasis: A murine model of breast cancer. *PLoS ONE*, *8(9)*: 74753. DOI: 10.1371/journal.pone.0074753.

Abdullah, N A; Mahiyuddin, W R W; Muhammad, N A; Ali, Z M; Ibrahim, L; Tamim, N S I; Mustafa, A N and Kamaluddin, M A (2013). Survival rate of breast cancer patients in Malaysia: A population-based study. *Asian Pac. J. Cancer Prev.*, *14(8)*: 4591-4594. DOI: 10.7314/APJCP.2013.14.8.4591.

Abraham, A; Kattoor, A J; Saldeen, T and Mehta, J L (2019). Vitamin E and its anticancer effects. *Crit. Rev. Food Sci. Nutr.*, *59(17)*: 2831-2838. DOI: 10.1080/10408398.2018.1474169.

Abuasal, B S; Qosa, H; Sylvester, P W and Kaddoumi, A (2012). Comparison of the intestinal absorption and bioavailability of γ-tocotrienol and α-tocopherol: *In vitro, in situ* and *in vivo* studies. *Biopharm. Drug Dispos.*, *33(5)*: 246-256. DOI: 10.1002/bdd.1790.

Aggarwal, V; Kashyap, D; Sak, K; Tuli, H S; Jain, A; Chaudhary, A; Garg, V K; Sethi, G and Yerer, M B (2019). Molecular mechanisms of action of tocotrienols in cancer: Recent trends and advancements. *Int. J. Mol. Sci.*, *20(3)*: 656. DOI: 10.3390/ijms20030656.

Alizadeh, D; Wong, R A; Gholamin, S; Maker, M; Aftabizadeh, M; Yang, X; Pecoraro, J R; Jeppson, J D; Wang, D; Aguilar, B; Starr, R; Larmonier, C B; Larmonier, N; Chen, M-H; Wu, X; Ribas, A; Badie, B; Forman, S J and Brown, C E (2021). IFNg is critical for CAR T cell mediated myeloid activation and induction of endogenous immunity. *Cancer Discov.*, *11(9)*: 2248-2265. DOI: 10.1158/2159-8290.CD-20-1661.

Azizah, A M; Hashimah, B; Nirmal, K; Siti Zubaidah, A R; Puteri, N A; Nabihah, A; Sukumaran, R; Balqis, B; Nadia, S M R; Sharifah, S S S; Rahayu, O; Nur Alham, O and Azlina, A A (2019). *Malaysia National Cancer Registry Report (MNCR) (2012-2016)*. Ministry of Health, Putrajaya. p. 1-100.

Bray, F; Ferlay, J; Soerjomataram, I; Siegel, R L; Torre, L A and Jemal, A (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer J. Clinicians*, *68(6)*: 394-424. DOI: 10.3322/caac.21492.

De Silva, L; Chuah, L H; Meganathan, P and Fu, J Y (2016). Tocotrienol and cancer metastasis. *Biofactors*, *42(2)*: 149-162. DOI: 10.1002/biof.1259.

Fedchenko, N and Reifenrath, J (2014). Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue - A review. *Diagn. Pathol.*, *9(1)*: 221. DOI: 10.1186/s13000-014-0221-9.

Fontana, F; Raimondi, M; Marzagalli, M; Audano, M; Beretta, G; Procacci, P; Sartori, P; Mitro, N and Limonta, P (2020). Mitochondrial functional and structural impairment is involved in the antitumor activity of δ-tocotrienol in prostate cancer cells. *Free Radic. Biol. Med.*, *160*: 376-390. DOI: 10.1016/j.freeradbiomed.2020.07.009.

Ghanem, P; Zouein, A; Mohamad, M; Hodroj, M H; Haykal, T; Abou Najem, S; Naim, H Y and Rizk, S (2019). The vitamin E derivative gamma tocotrienol promotes anti-tumor effects in acute myeloid leukemia cell lines. *Nutrients*, *11(11)*: 2808. DOI: 10.3390/nu11112808.

Hafid, S R A; Radhakrishnan, A K and Nesaretnam, K (2010). Tocotrienols are good adjuvants for developing cancer vaccines. *BMC Cancer*, *10(1)*: 5. DOI: 10.1186/1471-2407-10-5.

Huang, Y; Ma, C; Zhang, Q; Ye, J; Wang, F; Zhang, Y; Hunborg, P; Varvares, M A; Hoft, D F; Hsueh, E C and Peng, G (2015). $CD4^+$ and $CD8^+$ T cells have opposing roles in breast cancer progression and outcome. *Oncotarget, 6(19)*: 17462-78. DOI: 10.18632/oncotarget.3958.

Kabir, M I; Adnan, M and Rahman, M M (2017). Natural sources of tocotrienols: A note on absorption. *J. In Silico* In vitro *Pharmacol.*, *3*: 20. DOI: 10.21767/2469-6692.100020.

Knochelmann, H M; Dwyer, C J; Bailey, S R; Amaya, S M; Elston, D M; Mazza-McCrann, J M and Paulos, C M (2018). When worlds collide: Th17 and Treg cells in cancer and autoimmunity. *Cell. Mol. Immunol.*, *15(5)*: 458-469. DOI: 10.1038/s41423-018-0004-4.

Maarasyid, C; Muhamad, I I and Supriyanto, E (2014). Potential source and extraction of vitamin E from palm-based oils: A review. *Jurnal Teknologi*, *69(4)*. DOI: 10.11113/jt.v69.3172.

Mba, O I; Dumont, M J and Ngadi, M (2015). Palm oil: Processing, characterization and utilization in the food industry - A review. *Food Biosci.*, *10*: 26-41. DOI: 10.1016/j.fbio.2015.01.003.

Medeiros, B and Allan, A L (2019). Molecular mechanisms of breast cancer metastasis to the lung: Clinical and experimental perspectives. *Int. J. Mol. Sci.*, *20(9)*: 2272. DOI: 10.3390/ijms20092272.

Mikami, N; Kawakami, R and Sakaguchi, S (2020). New Treg cell-based therapies of autoimmune diseases: Towards antigen-specific immune suppression. *Curr. Opin. Immunol.*, *67*: 36-41. DOI: 10.1016/j.coi.2020.07.004.

Montagnani Marelli, M; Marzagalli, M; Fontana, F; Raimondi, M; Moretti, R M and Limonta, P (2019). Anticancer properties of tocotrienols: A review of cellular mechanisms and molecular targets. *J. Cell. Physiol.*, *234(2)*: 1147-1164. DOI: 10.1002/jcp.27075.

Nesaretnam, K; Selvaduray, K R; Abdul Razak, G; Veerasenan, S D and Gomez, P A (2010). Effectiveness of tocotrienol-rich fraction combined with tamoxifen in the management of women with early breast cancer: A pilot clinical trial. *Breast Can. Res.*, *12(5)*: 1-8. DOI: 10.1186/bcr2726.

Nesaretnam, K; Stephen, R; Dils, R and Darbre, P (1998). Tocotrienols inhibit the growth of human breast cancer cells irrespective of estrogen receptor status. *Lipids*, *33(5)*: 461-469. DOI: 10.1007/s11745-998-0229-3.

Nesaretnam, K; Guthrie, N; Chambers, A F and Carroll, K K (1995). Effect of tocotrienols on the growth of a human breast cancer cell line in culture. *Lipids*, *30(12)*: 1139-1143. DOI: 10.1007/BF02536615.

Ohue, Y and Nishikawa, H (2019). Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? *Cancer Sci.*, *110(7)*: 2080-2089. DOI: 10.1111/cas.14069.

Ording, A G; Heide-Jørgensen, U; Christiansen, C F; Nørgaard, M; Acquavella, J and Sørensen, H T (2017). Site of metastasis and breast cancer mortality: A Danish nationwide registry-based cohort study. *Clin. Exp. Metastasis*, *34(1)*: 93-101. DOI: 10.1007/s10585-016-9824-8.

Panneerselvam, J; Srivastava, A; Mehta, M; Chen, A; Zhao, Y D; Munshi, A and Ramesh, R (2019). IL-24 inhibits lung cancer growth by suppressing GLI1 and inducing DNA damage. *Cancers*, *11(12)*: 1879. DOI: 10.3390/cancers11121879.

Pulaski, B A and Ostrand-Rosenberg, S (2000). Mouse 4T1 breast tumor model. *Curr. Protoc. Immunol.*, *39(1)*: 20.2.1-20.2.16. DOI: 10.1002/0471142735.im2002s39.

Rzepecka, A; Żmigrodzka, M; Witkowska-Piłaszewicz, O; Cywińska, A and Winnicka, A (2019). CD4 and MHCII phenotypic variability of peripheral blood monocytes in dogs. *PLoS ONE*, *14(7)*: e0219214. DOI: 10.1371/journal.pone.0219214.

Sazli, F A R; Jubri, Z; Rahman, M A; Karsani, S A; Top, A G M and Ngah, W Z W (2015). Gamma-tocotrienol treatment increased peroxiredoxin-4 expression in HepG2 liver cancer cell line. *BMC Complement Altern. Med.*, *15(1)*: 64. DOI: 10.1186/s12906-015-0590-y.

Sambucci, M; Gargano, F; Guerrera, G; Battistini, L and Borsellino, G (2019). One, no one, and one hundred thousand: T regulatory cells' multiple identities in neuroimmunity. *Front. Immunol.*, *10*: 2947. DOI: 10.3389/fimmu.2019.02947.

Sasidharan Nair, V and Elkord, E (2018). Immune checkpoint inhibitors in cancer therapy: A focus on T-regulatory cells. *Immunol. Cell Biol.*, *96(1)*: 21-33. DOI: 10.1111/imcb.1003.

Selvaduray, K R; Radhakrishnan, A K; Kutty, M K and Nesaretnam, K (2012). Palm tocotrienols decrease levels of pro-angiogenic markers in human umbilical vein endothelial cells (HUVEC) and murine mammary cancer cells. *Genes Nutr.*, *7(1)*: 53-61. DOI: 10.1007/s12263-011-0223-0.

Selvaduray, K R; Radhakrishnan, A K; Kutty, M K and Nesaretnam, K (2010). Palm tocotrienols inhibit proliferation of murine mammary cancer cells and induce expression of interleukin-24 mRNA. *J. Interferon Cytokine Res.*, *30(12)*: 909-916. DOI: 10.1089/jir.2010.0021.

Shaharudin, S H; Sulaiman, S; Emran, N A and Shahril, M R (2011). The use of complementary and alternative medicine among Malay breast cancer survivors. *Alt. Ther. Health Med.*, *17(1)*: 50.

Srivastava, J K and Gupta, S (2006). Tocotrienol-rich fraction of palm oil induces cell cycle arrest and apoptosis selectively in human prostate cancer cells. *Biochem. Biophys. Res. Commun.*, *346(2)*: 447-453. DOI: 10.1016/j.bbrc.2006.05.147.

Subramaiam, H; Chu, W L; Radhakrishnan, A K; Chakravarthi, S; Selvaduray, K R and Kok, Y Y (2021). Evaluating anticancer and immunomodulatory effects of spirulina (Arthrospira) platensis and gamma-tocotrienol supplementation in a syngeneic mouse model of breast cancer. *Nutrients*, *13(7)*: 2320. DOI: 10.3390/nu13072320.

Tanaka, A and Sakaguchi, S (2019). Targeting Treg cells in cancer immunotherapy. *Eur. J. Immunol.*, *49(8)*: 1140-1146. DOI: 10.1002/eji.201847659.

Verma, A; Mathur, R; Farooque, A; Kaul, V; Gupta, S and Dwarakanath, B S (2019). T-regulatory cells in tumor progression and therapy. *Cancer Manag. Res.*, *11*: 10731-10747. DOI: 10.2147/CMAR.S228887.

Wada, S; Naito, Y; Matsushita, Y; Nouchi, M; Kawai, M; Minami, E; Aoi, W; Ikeda, S; Higashi, A and Yoshikawa, T (2017). δ-tocotrienol suppresses tumorigenesis by inducing apoptosis and blocking the COX-2/PGE2 pathway that stimulates tumor-stromal interactions in colon cancer. *J. Funct. Foods*, *35*: 428-435. DOI: 10.1016/j.jff.2017.06.002.

Weng-Yew, W; Selvaduray, K R; Ming, C H and Nesaretnam, K (2009). Suppression of tumor growth by palm tocotrienols via the attenuation of angiogenesis. *Nutr. Cancer*, *61(3)*: 367-373. DOI: 10.1080/01635580802582736.

Wu, S J and Ng, L T (2010). Tocotrienols inhibited growth and induced apoptosis in human HeLa cells through the cell cycle signaling pathway. *Integr. Cancer Ther.*, *9(1)*: 66-72. DOI: 10.1177/1534735409357757.

Yamamoto, K; Kobayashi, H; Miura, O; Hirosawa, S and Miyasaka, N (1997). Assignment (a) of IL12RB1 and IL12RB2, interleukin-12 receptor (beta) 1 and (beta) 2 chains, to human chromosome 19 band p13. 1 and chromosome 1 band p31. 2, respectively, by *in situ* hybridization. *Cytogenet. Cell Genet.*, *77(3-4)*: 257-258. DOI: 10.1159/000134589.

Yu, N; Li, X; Song, W; Li, D; Yu, D; Zeng, X; Li, M; Leng, X and Li, X (2012). $CD4^+$ $CD25^+$ CD127 low/– T cells: A more specific Treg population in human peripheral blood. *Inflammation*, *35(6)*: 1773-1780. DOI: 10.1007/s10753-012-9496-8.

Zhang, Y; Liu, Y and Xu, Y (2019). Interleukin-24 regulates T cell activity in patients with colorectal adenocarcinoma. *Front. Oncol.*, *9*: 1401. DOI: 10.3389/fonc.2019.01401.

Zhang, Y H; Ma, K; Liu, J R; Wang, H X; Tian, W X; Tu, Y H and Sun, W G (2018). γ-tocotrienol inhibits the invasion and migration of human gastric cancer cells through downregulation of cyclooxygenase-2 expression. *Oncol. Rep.*, *40(2)*: 999-1007. DOI: 10.3892/or.2018.6497.

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EFFECT OF SODIUM CASEINATE/CELLULOSE NANOCRYSTALS ADDITION ON THE PHYSICAL AND OXIDATIVE STABILITY OF RED PALM OLEIN-IN-WATER PICKERING EMULSIONS

SEE KIAT WONG[1]; CHIEN LYE CHEW[1,2]; JANARTHANAN SUPRAMANIAM[1]; BENG TI TEY[1,3]; TIN WUI WONG[4] and SIAH YING TANG[1,3,5]*

ABSTRACT

*This work investigated the use of a dual biopolymer system containing cellulose nanocrystals (CNC) and sodium caseinate (SC) for red palm olein-in-water (O/W) Pickering emulsion stabilisation. The effect of CNC particle concentration at a constant amount of SC on emulsion properties was particularly studied. The resultant mixed biopolymer-based emulsions were characterised for droplet size distribution, zeta potential, creaming index and microstructure, as well as primary and secondary oxidation products by oxidation tests at 90°C. Results showed that creaming stability was relatively high for SC/CNC-stabilised emulsions compared to emulsions prepared with SC alone. Increasing CNC concentration from 0.25% to 1.00% (w/v) resulted in a gradual decrease in emulsion droplet size with increased surface negative charges. Higher CNC content induced a marked reduction in the lipid oxidation of SC/CNC-stabilised emulsions. The mixed SC/CNC biopolymer significantly improved the carotene retention of emulsions during the accelerated oxidation test. Emulsions comprising 1.00% SC and 1.00% CNC presented the highest negative surface charge, lowest peroxide value (PV), p-anisidine value (*p*-AV), free fatty acid (FFA) value, and total oxidation value (TOTOX). The use of CNC imparted beneficial effects on reducing lipid oxidation and enhancing the stability of palm-based O/W emulsions stabilised by SC.*

Keywords: cellulose nanocrystals, oxidative stability, physical stability, Pickering emulsions, sodium caseinate.

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[1] Chemical Engineering Discipline, School of Engineering, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, 47500 Subang Jaya, Selangor, Malaysia.

[2] Sime Darby Research, R&D Centre, Carey Island, Lot 2664, Jalan Pulau Carey, 42960 Carey Island, Selangor, Malaysia.

[3] Advanced Engineering Platform, School of Engineering, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, 47500 Subang Jaya, Selangor, Malaysia.

[4] Non-Destructive Biomedical and Pharmaceutical Research Centre, Smart Manufacturing Research Institute, Universiti Teknologi MARA, 42300 Puncak Alam, Selangor, Malaysia.

[5] Tropical Medicine and Biology Platform, School of Science, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, 47500 Subang Jaya, Selangor, Malaysia.

* *Corresponding author email: patrick.tang@monash.edu*

INTRODUCTION

Lipid oxidation is when free radicals 'attack' and 'steal' electrons from lipid molecules, causing oxidative degradation of lipid. This could lead to the poor stability and shorter shelf-life of lipid-containing emulsion products. It is an undesirable process that leads to adverse impacts on food products, lowering overall quality. Oxidative rancidity, nutrients denaturation and potential toxicity are some of the unfavourable outcomes caused by lipid oxidation (Jacobsen *et al*., 2013). Generally, oils and fats that contain long-chain polyunsaturated fatty acids are more vulnerable to oxidation during storage or heat treatment as they have multiple double bonds and reactive hydrogen atoms. Especially oil-in-water (O/W) emulsions

are thermodynamically unstable systems that are more vulnerable to lipid oxidation because the oil globules have an overall higher interfacial area than bulk oils (Berton-Carabin *et al.*, 2014). The larger specific surface area allows more oxidation reactions to occur, resulting in a faster lipid oxidation rate that decreases the stability of the food products (Hu *et al*., 2016).

Red palm olein contains a higher level of carotenes and antioxidants than traditional refined palm oil because it is only mildly processed, allowing it to retain the nutritional compounds (Bonnie Tay and Choo, 2000). Low *et al.* (2017) successfully fabricated red palm olein-in-water (O/W) Pickering emulsions with dual stimulus responses using magnetic cellulose nanocrystals (CNC) as the stabiliser. Most recently, Wong *et al*. (2021) demonstrated the preparation of highly stable red palm olein-based Pickering emulsions using CNC-soy protein isolate nanoconjugates. Even though red palm olein possesses high antioxidant content, lipid oxidation is often unavoidable in the case of O/W emulsions. Lipid oxidation is believed to occur at the O/W interface (Liu *et al*., 2018), where reactive oxygen species are readily available. LOOH and aldehydes or ketones are generated as the primary and secondary oxidation products, respectively (Xu *et al*., 2017b). These molecules eventually generate dietary advanced lipid oxidation end-products, threatening human health when consumed (Cao *et al*., 2018). This challenges researchers to seek desirable ingredients to stabilise such a food emulsion system using surfactants and colloidal particles.

Synthetic surfactants such as sorbitan esters and their ethoxylates and sucrose esters are commonly used in forming food emulsions. Still, research has demonstrated that the accumulative use of surfactants could result in toxicity and carcinogenicity. Thus, selecting suitable emulsifiers in food applications is still limited due to their non-biocompatible properties (Ho *et al*., 2016). Recently, many efforts have been invested in using food-grade colloidal particles to stabilise food-based emulsions. Incorporating colloidal particles to shield the oil and form a layer or multiple layers around the oil droplets can enhance its stability. The colloidal particles that are adsorbed irreversibly at the oil-water interface provide high stability to the emulsion as an energy barrier that effectively separates the two immiscible phases (Zoppe *et al*., 2012). This emulsion system is known as Pickering emulsion and has been widely used in biomedical, food and cosmetic products. Pickering emulsifiers have demonstrated a better stabilisation effect against coalescence of emulsified droplets, and some solid particles also possess additional properties such as antioxidants, antimicrobial and responsiveness to external stimulus (Ho *et al*., 2016). Hence, selecting suitable food-grade solid particles to form an edible emulsion is crucial in producing products with desired properties and outstanding stability.

As one of the most promising and environmentally friendly particles, sodium caseinate (SC) has been utilised as a biopolymer emulsifier due to its lower cost, improved stabilisation and oxidation stability than other milk proteins (whey protein isolate, calcium caseinate and β-lactoglobulin) (Chang and Nickerson, 2018). However, proteins are vulnerable to changes in temperature, pH and ionic strength, which limit their applications, especially in the food industry (Hu *et al*., 2016). Hence, a secondary colloidal particle such as a polysaccharide may be introduced into the formulation to enhance the emulsion stability and reduce the oxidation rate.

CNC have attracted much attention as a food-grade material source due to their abundant availability, biocompatibility, renewability, and flexibility with surface modification to increase their desired characteristics (Zoppe *et al.,* 2012). CNC was commonly produced by sulphuric acid hydrolysis, which yields rod-like crystals with unique surface charges and mechanical properties. CNC has been considered an excellent Pickering stabiliser for food emulsion due to its non-toxicity, anti-oxidative properties, biodegradability and abundant supply (Low *et al*., 2019; Wong *et al*., 2021). CNC utilisation as a stabiliser in Pickering emulsion is expected to bring more advantages to the emulsion system. Studies have shown that the combination of proteins and polysaccharides as the stabiliser improved the oxidative stability and physical stability of emulsions formed (Li *et al*., 2017; Xu *et al*., 2017a). Previous studies have shown that the SC/CNC-stabilised emulsions were formed due to the higher surface activity of SC and irreversibility of adsorption of CNC at pH 7 (Pinďáková *et al*., 2019). However, the use of SC/CNC biopolymeric mixture has not been explored in greater depth for both physical and oxidative stability improvement of palm-olein-based Pickering emulsions in the literature.

The present study aimed to investigate the addition of different CNC concentrations with SC on emulsion stabilisation processes. For this purpose, red palm O/W Pickering emulsions were prepared and stabilised by SC solely or mixed with CNC particles. The influence of different CNC concentrations [from 0.25% to 1.00% (w/v)] at a fixed amount of SC (1.00% w/v) on emulsion physical and oxidative stability was investigated. The physical stability parameters such as creaming index, droplet size, zeta potential and microstructures of resultant emulsions were measured, and analysed. The overall oxidative

stability of the encapsulated red palm olein was evaluated by determining the peroxide value (PV), *p*-anisidine value (*p*-Av), free fatty acid (FFA) value, total oxidation value (TOTOX), and carotene content via accelerated oxidation test at 90°C.

MATERIALS AND METHODS

Materials

SC was purchased from Sigma Chemicals Co. (St. Loius, MO, USA). CNC (freeze-dried, 0.96 wt% sulphur content) were procured from the University of Maine, USA. Red palm superolein was acquired from Sime Darby Jomalina Sdn. Bhd., Malaysia. Ultrapure water (18.2 MΩ cm^{-1}) was obtained from the Milli-Q® Plus apparatus (Millipore, Billerica, USA). All chemicals used in this study were of analytical grade.

Preparation of Olein-in-Water (O/W) Emulsions

A series of emulsions were prepared using the ultrasonication method (all in weight proportion). Briefly, SC (1.00% w/v) was first dispersed in deionised water using a magnetic stirring apparatus at room temperature for 2 hr. The solution was stored overnight at 4°C before Pickering emulsion preparation. The continuous aqueous phase with four different CNC concentrations was prepared using CNC particles of 0.25%, 0.50%, 0.75% and 1.00% (w/v) into the prepared SC solution followed by uniform mixing. The oil phase, namely palm olein (20%), was gradually added into the resultant polysaccharide-protein mixtures and emulsified using ultrasonic horn (20 kHz, 50W system, NexTgen ultrasonic platform, Sinaptec, France) at room temperature for 5 min. The resultant emulsions were transferred to glass vials and stored at room temperature in the dark for further analysis. During the emulsion preparation, sodium azide of 0.02% (w/v) was added to control microbial growth during storage. Emulsion stabilised by SC alone (0.00% CNC) was used as the control throughout the whole study.

Characterisation of Biopolymers

Particle size and zeta potential. The average particle size of SC, CNC and mixed SC/CNC samples were measured using dynamic light scattering (DLS) (Zetasizer Nano, Malvern Instruments, United Kingdom). The surface charge was measured in terms of zeta potential using the laser Doppler micro-electrophoresis technique (Zetasizer Nano, Malvern Instruments, United Kingdom) at pH 7.5 and 25°C. Triplicates were conducted for each sample, and the results were averaged.

Morphology of particles. The size and morphology of SC, CNC and mixed SC/CNC samples were analysed using a field emission scanning electron microscope (FE-SEM) (Hitachi, SU8010, Japan) at 15 kV in STEM mode. The samples were prepared by dispersing in deionised water and sonicated for 5 min. Then, the samples were dipped onto a 300-mesh formvar carbon-coated copper grid. The copper grids were air-dried before viewing.

Water contact angle. The water contact angle was measured using a sessile drop method with a goniometer (Model 190, Rame-hart, USA). Briefly, microscope glass slides were coated with mixtures containing SC with different CNC concentrations by depositing the freshly sonicated suspension onto the clean substrates and dried at 50°C overnight. The water contact angle measurement was performed by placing a drop of water at five different points on the SC-CNC-coated thin film using a precision micro-syringe. Then, water contact angles were determined by applying the Laplace-Young fitting algorithm. The result was reported as the average of these five-point measurements.

Characterisation of Pickering Emulsion

Creaming index (CI). The emulsions were prepared, placed in vials hermetically, and stored upright at 25°C. The CI was measured via visual inspection of the emulsion separation into an upper oil-rich layer, cream layer and a lower oil-depleted or serum layer at days 0 and 30 during storage. The CI was determined using the Equation (1):

$$CI\ (\%) = H_C/H_T \quad (1)$$

where, H_c = height of the cream layer and H_T = total height of the emulsion.

Droplet size and zeta potential. The mean droplet size ($D_{4,3}$) and size distribution of all samples were measured by static light scattering (Mastersizer 3000, Malvern Instrument, United Kingdom) equipped with a Hydro EV wet dispersion unit. Deionised water was used as the dispersant. The refractive index of palm olein and dispersant phase are 1.458 and 1.330, respectively. The droplet size of all the emulsion samples was measured in triplicate, and results were reported as volume-weighted mean diameter, $D_{4,3}$. The zeta potential of all samples was analysed via laser Doppler micro-electrophoresis technique (Zetasizer NanoZS 90, Malvern Instrument, United Kingdom). Each sample was diluted

100 times with deionised water before measurement. The result was reported as the average of three measurements.

Microstructural analysis. The morphological structures of the emulsions were determined using an inverted optical microscope (Nikon Eclipse TS100, Nikon Instruments Inc., USA) at 20x magnification. A 10 µL of each emulsion was placed on a glass, and then, a coverslip was carefully placed to prevent the trapping of air bubbles. The images were then captured using a charge-coupled device (CCD) camera.

Analysis of lipid oxidation in emulsions. The oxidative stability measurements of PV, *p*-AV, FFA, TOTOX and carotene content were performed throughout five days of the accelerated oxidation study. The oil extraction method was adopted using a mixture of isopropanol and hexane (1:3, v/v) to break the emulsions. The resultant solution was vortexed for 2 min, and the organic layer was separated via centrifugation at 4000 rpm for 20 min, followed by rotary evaporation. The extracted oil (red palm olein) was collected and kept for subsequent analysis**.**

Primary oxidation products - peroxide value (PV). Lipid hydroperoxides (LOOH) were measured as primary oxidation products according to the American Oil Chemist's Society (AOCS) official methods, Cd 8b-90 (AOCS, 1997). Briefly, 3 g of oil sample was added to a mixture of 30 mL glacial acetic acid/ chloroform (3:2 v/v). Subsequently, 0.5 mL of saturated potassium iodide solution was added, and the resultant mixture was vortexed for 1 min. A total of 30 mL of deionised water was then added, followed by 0.5 mL of a starch solution as the indicator. The final mixture was titrated against 0.01 N sodium thiosulphate until the indicator changed its colour from blue-black to colourless. The PV was determined using the Equation (2):

$$PV = \frac{(V \times N) \times 1000}{W} \quad (2)$$

where, V = volume in mL of sodium thiosulphate solution used, N = normality of sodium thiosulphate solution, and W = weight in grams of the test portion. Measurements were carried out in triplicate.

***Secondary oxidation products* - p-*anisidine value* (p-*AV*).** Secondary oxidation products, namely aldehydes and ketones associated with oxidative rancidity were measured according to AOCS official methods, Cd 18-90 (AOCS, 1997). Briefly, 1 g of oil sample was added into a volumetric flask, and the volume was made up to the mark with iso-octane as the solvent. The absorbance was then measured using a UV-visible spectrophotometer (Varian Cary 50, USA) at 350 nm and used as blank. A 5 mL of oil sample was added with 1 mL of *p*-anisidine, and the resultant solution was mixed thoroughly. The absorbance of the sample treated with p-anisidine was recorded at 350 nm after 10 min. The *p*-AV was determined using the Equation (3):

$$p\text{-AV} = \frac{100 \times Q \times V \times [1.2(As - Ab)]}{W} \quad (3)$$

where, A_s = absorbance of sample added with *p*-anisidine at 350 nm, A_b = absorbance of the sample without p-anisidine at 350 nm, Q = sample content in grams per millilitres (Q = 0.1 g mL^{-1}) of the measured solution based on which the anisidine value is expressed, V = volume in grams per millilitres (V = 25 mL) in which, the sample is dissolved, 1.2 = correction factor for the dilution of the 5 mL of the test solution with 1 mL of the p-anisidine and W = weight in grams (g) of the test portion.

Free Fatty Acid (FFA)

The acidity test was conducted according to AOCS official methods, Ca 5a-40 (AOCS, 1997). Briefly, about 5-10 g of oil sample were added into 50 mL of heated isopropanol: phenolphthalein (100:1, v/v%). A 0.1 M potassium hydroxide was added dropwise into the resultant mixture until a faint but permanent pink colour was obtained. The neutralised mixture was added into the 250 mL conical flask and titrated using 0.1 M potassium hydroxide until a permanent faint pink colour was obtained. The FFA was then determined using the Equation (4):

$$\text{FFA (palmitic acid)} = \frac{25.6 \times M \times V}{W} \quad (4)$$

where M = molarity of potassium hydroxide solution, V = volume in mL of potassium hydroxide solution used, and W = weight in grams (g) of the test portion.

Total oxidation value (TOTOX) and carotene content. The TOTOX and carotene content are essential measurements for oxidation study in palm olein. Generally, the lower the TOTOX value, the better the quality of the oil. Carotene content is another key parameter in protecting the oil quality, given its pro-antioxidant property. The determination of both TOTOX and carotene content was performed according to Malaysian Palm Oil Board (MPOB) test methods, p2.14 and p2.6 (Ainie *et al*., 2004), respectively. Briefly, 0.1 g of oil sample was added into a volumetric flask, and the volume

was made up to the mark with iso-octane as the solvent. The TOTOX value and carotene value were calculated using the Equations (5) and (6), respectively:

$$\text{TOTOX} = \text{Corrected } A_{233} + \text{Corrected } A_{446} \quad (5)$$

where, Corrected $A_{233} = A_{233} - \frac{0.06}{383} \times$ carotene content in mg/kg and Corrected $A_{446} = A_{446} - \frac{0.06}{383} \times$ carotene content in mg/kg

$$\text{Carotene (mg/kg)} = \frac{383 \times A}{l \times C} \quad (6)$$

where 383 = the extinction coefficient for carotenoids, A = absorbance of the sample at 269 nm, l = path length of the cell (in cm), and C = concentration of sample used for measurement (in g per 100 mL).

Statistical analysis. All the results were expressed as the mean value ± standard deviation of the measurements. Analysis of variance (ANOVA) was conducted using Prism software, and the differences were considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

Characterisation of SC, CNC and SC/CNC Mixture

Particle size is an essential characteristic for Pickering emulsion stabiliser, as it directly affects the emulsion droplet size. Smaller particles can assemble quickly around the oil-water interfaces to facilitate the formation of smaller oil droplets (Song *et al*., 2020). SC exhibited the largest average particle size (*Figure 1a*) at 334.1 ± 20.6 nm, which decreased to 256.9 ± 35.4 nm after the addition of CNC at a 1:1 ratio (w/w). The reduction in particle size could be due to the presence of smaller CNC particles (127.4 ± 12.1 nm), contributing to a shift in particle size distribution to a lower region (smaller size) in the DLS analysis.

The microstructure of SC, CNC and mixed SC/CNC was evaluated by scanning transmission electron microscopy (STEM), as shown in *Figures 1b, 1c* and *1d*. Micrographs of SC particles alone showed a mixture of non-uniform aggregated particles with some spherical globular proteins. The particle size obtained from STEM analysis displayed a wide range of sizes, with the smallest size at <100 nm and the biggest size (aggregates) at >300 nm. However, the results obtained by STEM were not in good agreement with the results of our DLS analysis. The observed difference in particle sizes could be due to the occurrence of aggregation of SC particles when dried from a monodispersed suspension, which rendered a larger average particle size of around ~334 nm. Nevertheless, the results agreed with other reported values between 50-600 nm (Francis *et al*., 2019; Le *et al*., 2008). On the other hand, CNC particles showed needle-like structures with a length of 100-150 nm and a diameter of <10 nm. The mixed SC/CNC micrograph displayed two distinct shaped particles with the larger globular particles as SC and the smaller needle-like particles as CNC. No morphological or structural changes of SC particles were observed upon the addition of CNC.

The surface charge is another critical indicator for measuring the stability of emulsion droplets. The mixed SC/CNC biopolymer possessed good stability with a zeta potential of -37.7 ± 2.5 mV, whereas the recorded zeta potential of SC alone was -19.1 ± 0.8 mV at pH 7.5. SC has an isoelectric constant (pI) of 4.6, which will exist in its anionic form at a pH value greater than its pI (Zinoviadou *et al*., 2012). Zeta potential of ± 30 mV is considered the borderline for good colloidal stability (Luo *et al*., 2013). The addition of a more negatively charged species (CNC particles) increased the overall charges of the mixed biopolymer system, contributing to greater electrostatic repulsion when used as an emulsion stabiliser in combination.

To characterise the wettability of SC and mixed SC/CNC biopolymer with different CNC concentrations, contact angle measurements were investigated on thin films coated with a biopolymeric emulsifier. As shown in *Figure 1e*, the water contact angle of SC alone was 79.69° ± 2.14° at room temperature. After the addition of CNC, the contact angle decreased with increased CNC concentration due to the hydrophilic nature of CNC. There was no statistically significant difference in the contact angle of samples containing 0.25% to 0.75% of CNC, indicating incomplete surface coverage of CNC in the film. However, at 1% CNC, the contact angle achieved was 68.57° ± 2.24°. The wettability of these particle coated films was higher, with a slightly more hydrophilic surface. The data agreed with another study as reported elsewhere (Sukyai *et al*., 2018). Based on the literature, the contact angle at $15° < \theta < 90°$ is suitable to form O/W emulsions, where the contact angle of the SC/CNC mixture falls within the optimum range (Low *et al*., 2020; Ortiz *et al*., 2020). The use of mixed SC/CNC particles with slightly lower contact angle did not lead to poor emulsion stability, as evidenced by our droplet stability study as shown in *Figure 4*. It has been well documented that particles with greater hydrophilicity will promote the formation of O/W emulsions and *vice versa* (Clegg *et al*., 2016; Jeon and Hong, 2014). Earlier studies also reported that utilising a blend of emulsifiers can produce emulsions with greater stability than using a single emulsifier (Chen *et al*., 2018; Liu

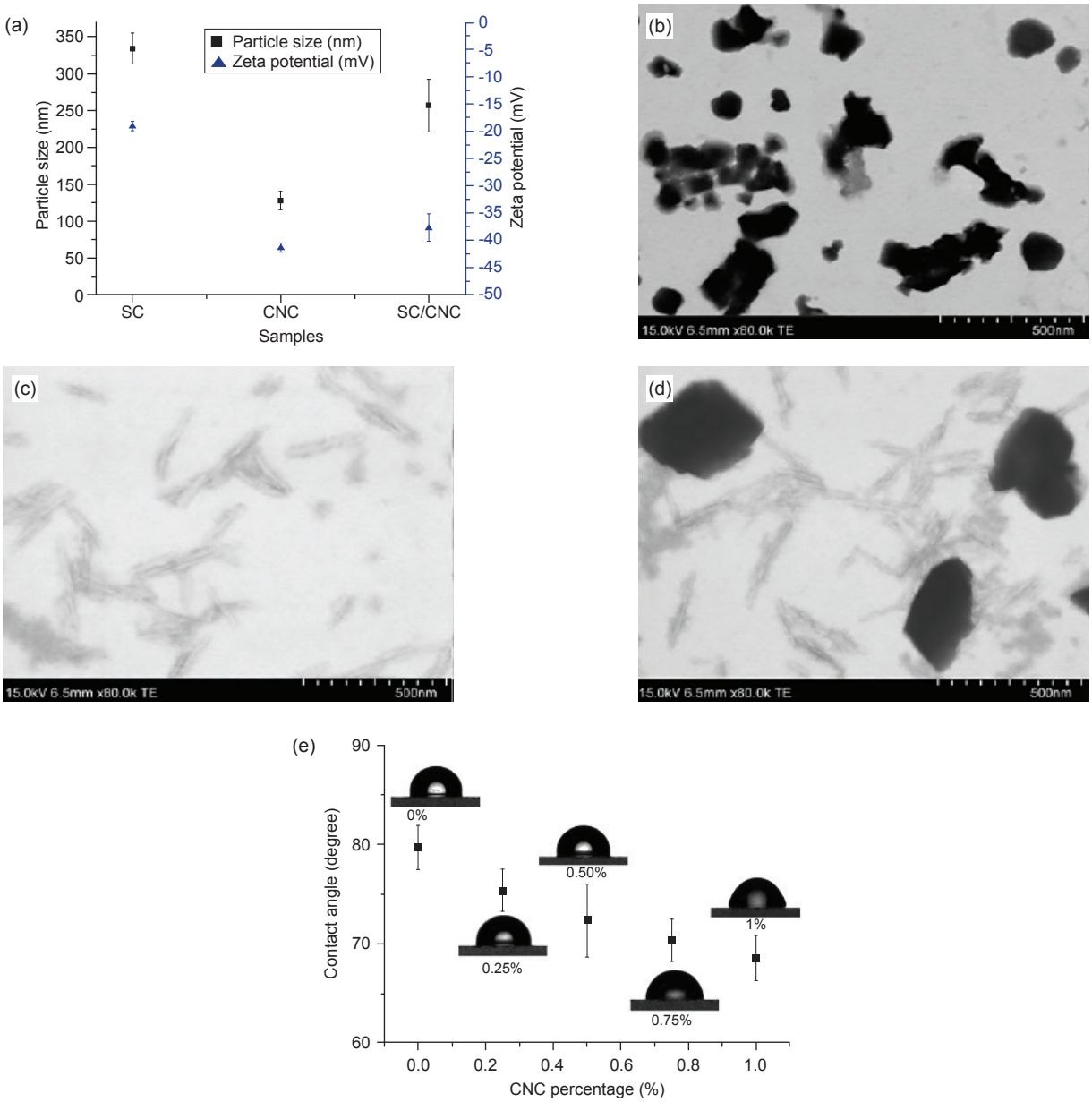

Figure 1. (a) Average particle size, zeta potential and micrographs of (b) SC, (c) CNC, (d) mixed SC/CNC, and (e) water contact angle of the aqueous solution containing 1.00% (w/v) SC and different CNC concentrations.

et al., 2012). The mixed SC/CNC was envisaged to demonstrate amphiphilic property, with SC particles preferentially wetted by the hydrophobic oil phase of the emulsion and the hydrophilic CNC particles by the aqueous phase.

Effect of CNC Content on Emulsion Stability, Particle Size and Zeta Potential

The stability of droplets in emulsions is generally conferred by a charged interface, which generates a strong electrostatic repulsion. These electrical properties of the interfacial layer are due to the presence of ionic groups (such as carboxyl, sulphate or phosphate groups) on the surface of the emulsifiers (McClements, 2015). Many food-grade amphiphilic polysaccharides (*e.g.*, modified cellulose, gum Arabic, and modified starch) are negatively charged. A combination of biopolymer emulsifiers can be applied to modulate the required electrical characteristics of emulsion droplets in different emulsion systems.

Emulsion stability was enhanced with the addition of CNC with SC, as shown in *Figure 2*. The maximum creaming indexes obtained for 0.00% CNC (contained SC alone) and formulation contained 1.00% CNC were 36.60% and 19.80%, respectively, at day 30, showing greater stability with 1.00% CNC as a reinforced stabiliser. The results demonstrated a lowered creaming index of the emulsions with increased CNC concentration. This can be deduced by the more robust three-

dimensional network formed between droplets by CNC particles, limiting the mobility of oil droplets and preventing aggregations (Hu *et al*., 2016). Phase separation occurred by migrating larger particles to the top of the vial because they contained a higher volume of less dense oil. In comparison, the smaller particles remained at the lower phase of the vial. It should be noted that the lower phase of the emulsions remained cloudy and opaque, with no visible phase separation, suggesting that the emulsion is stable throughout the 30-days storage. Research has shown that the more transparent and clearer the emulsion layer, the higher the degree of droplets flocculation and coalescence could have occurred (Manoj *et al*., 1998).

The $D_{4,3}$ of emulsions is shown in *Figure 3a*. There is a significant reduction between the average size of oil droplets stabilised by SC alone and 1.00% CNC with SC on the first day after the preparation of the emulsions. The average size of the emulsion droplets stabilised by SC alone (0.00% CNC) was ~68.00% bigger than oil droplets stabilised by mixed 1.00% SC/CNC. It was noted that there was a reduction of average droplet size with the addition of 0.25%-1.00% of CNC compared to 0.00% CNC on the day of preparation. However, no significant difference was observed in the average sizes of emulsions stabilised with all CNC concentrations at day 30. Storage stability study showed that the $D_{4,3}$ obtained for 0.00% CNC elevated from 12.70 ± 2.75 μm to 17.43 ± 2.57 μm (*Figure 3a*) over 30-days at room temperature. The particle size distribution showed a bimodal distribution where the second small peak has shifted to the right over the storage period, indicating coalescence of droplets into a larger one. From the microscopy images in *Figure 4*, the oil droplets stabilised by SC alone tended to aggregate into larger droplets, which was not found in the oil droplets stabilised by the mixed SC/CNC biopolymer. It was reported that the osmotic effect associated with the non-adsorbed casein molecules increased the attractive forces between the oil droplets, which accelerated droplets aggregation and coalescence rate of emulsions (Wang *et al*., 2018).

For formulation with added 1.00% CNC, the $D_{4,3}$ remained constant around 4.12 ± 0.32 μm throughout the entire storage period with similar particle size distribution (*Figure 4*). The $D_{4,3}$ of SC/CNC-stabilised emulsions was generally smaller than the emulsion containing SC alone. The difference in droplet diameter could be due to the higher volume of particles present during the emulsification process. More particles are available to absorb at the oil droplets interface, thus, forming smaller Pickering emulsion droplets. More colloidal particles are available to bind onto the oil and water interface during the emulsification process. Further aggregation was prevented presumably by steric repulsion and electrostatic forces by the absorbed particles (Guzey and McClements, 2006). Therefore, the SC/CNC-stabilised emulsions displayed better stability throughout the 30-days storage with minute changes in the $D_{4,3}$.

As depicted in *Figure 3b*, the ζ-potential of Pickering emulsions stabilised by SC alone was lower than the other formulations that contained different CNC concentrations. The ζ-potential of emulsion with 0.00% CNC decreased from -37.5 mV to -32.7 mV over 30-days storage at room temperature, showing some destabilisation of droplets when SC alone was used as the stabiliser. In contrast, the ζ-potential of the formulation containing 1.00% CNC was kept in the range of -50.0 mV to -53.0 mV throughout 30-days storage. These results indicated that the addition of CNC as a reinforced emulsifier increased the negative charges on the surface of the emulsion droplets, which consequently induced greater electrostatic repulsion between oil droplets to prevent flocculation and coalescence (Xu *et al*., 2017a). The high negatively charged surface of the SC/CNC-stabilised emulsion droplets corresponded to the constant $D_{4,3}$ throughout the 30-days storage as presented above.

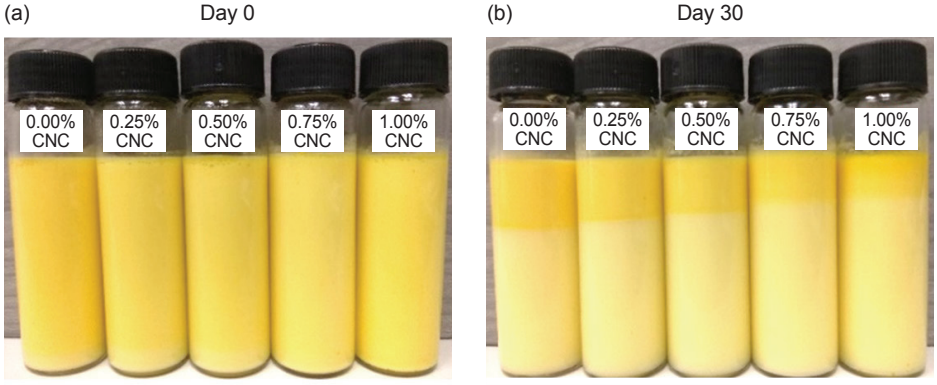

Figure 2. Creaming stability of O/W emulsion (20/80, v/v) with the aqueous phase containing 1.00% (w/v) of SC and different CNC concentrations at (a) day 0 and (b) day 30.

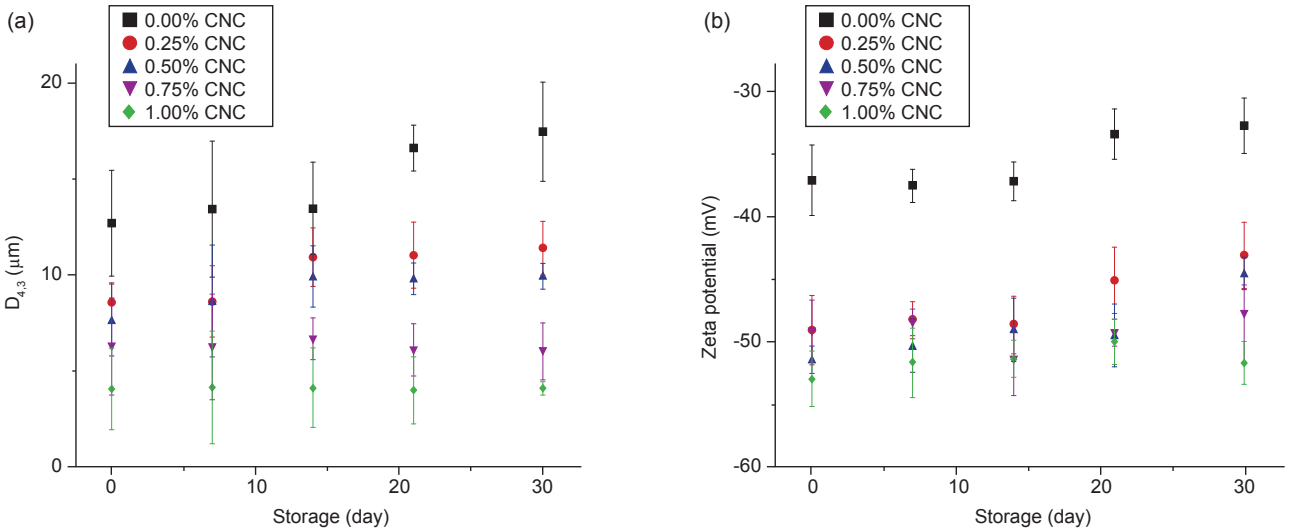

Figure 3. (a) Mean droplet size ($D_{4,3}$) and (b) zeta potential of O/W emulsion (20/80, v/v) with the aqueous phase containing 1.00% (w/v) of SC and different CNC concentrations as a function of storage time at 25°C. Data represent means (n=3) with standard deviations (error bars).

Influence of CNC Content on Lipid Hydroperoxide and *p*-Anisidine Value (*p*-AV)

The effect of different CNC concentrations on the oxidative stability of the O/W emulsions was assessed by measuring the PV and *p*-AV. PV and *p*-AV are used to calculate the estimation of primary and secondary oxidation of lipid, respectively. The comparison was made between the control group (0.00% CNC or containing SC alone) and the formulations containing different CNC concentrations. As depicted in *Figure 5a*, the PV content in 0.00% CNC and formulation contained 1.00% CNC, which has the highest content of CNC was approximately 1.307 and 1.310 meq kg^{-1} oil at day 0, and increased gradually with time to reach 38.25 and 27.79 meq kg^{-1} oil, respectively, after five days of accelerated oven testing at 90°C. By contrast, the amount of PV generated in SC/CNC-stabilised emulsions was significantly lower by 27% ($p<0.05$) than emulsions stabilised by SC alone, suggesting they were oxidised more slowly. The results also demonstrated that the oxidation trend of the emulsion was influenced by the concentration of CNC added, where an increase in CNC concentration generally lowered the generation of LOOH.

Similar results were observed in terms of secondary oxidation products as depicted in *Figure 5b*, the content of *p*-AV for 0.00% CNC and formulation contained 1.00% CNC was 3.180 and 3.147 at day 0, respectively. Then, the value raised to 4.581 and 3.698, respectively, after five days of accelerated testing. This revealed that the addition of CNC in forming emulsions could effectively reduce lipid oxidation, especially during secondary oxidation. The trend for developing LOOH and *p*-anisidine was similar for all the formulations containing mixed SC/CNC and 0.00% CNC during day 1 to day 4 of incubation. However, during the fifth day of incubation, the *p*-AV of the 0.00% CNC increased tremendously compared to other formulations. The rapid rise in *p*-AV that occurred later than that of LOOH could be explained as the primary oxidation products must decompose before forming the secondary oxidation products since the hydroperoxides are not stable at high temperature (Chew *et al.*, 2021). These results suggested that the SC/CNC-stabilised emulsions have a better antioxidant property than the formulation that contained SC alone. The results agreed with those reported elsewhere using a mix of polysaccharide and protein in stabilising O/W emulsion (Qiu *et al.*, 2015; Zeng *et al.*, 2017).

Lipid oxidation usually occurs more rapidly at the oil and water interface contact region in a biphasic system than bulk oil. In a biphasic system, the chain reaction of lipid peroxidation can be initiated more rapidly due to the close contact of pro-oxidants in the aqueous phase with the oil droplets (Lomova *et al.*, 2010). When a higher amount of solid particle emulsifiers is added, as in the case of increasing CNC concentration (0.25%-1.00%), emulsions are stabilised by droplet rich network surrounded by the solid particle emulsifiers which irreversibly adsorbed to oil-water interface (Zoppe *et al.*, 2012). This resulted in reduced lipid oxidation as displayed by lower PV and *p*-AV values. The colloid particles formed an energy barrier around the oil droplets which effectively blocked the highly reactive free radicals, as well as blocking the decomposition of LOOH into alkoxyl ($LO^{\cdot}$) and peroxyl ($LOO^{\cdot}$) radicals. Furthermore, lipid oxidation can be further retarded by reducing the activity of free radicals in accepting hydrogen from unsaturated fatty acids to

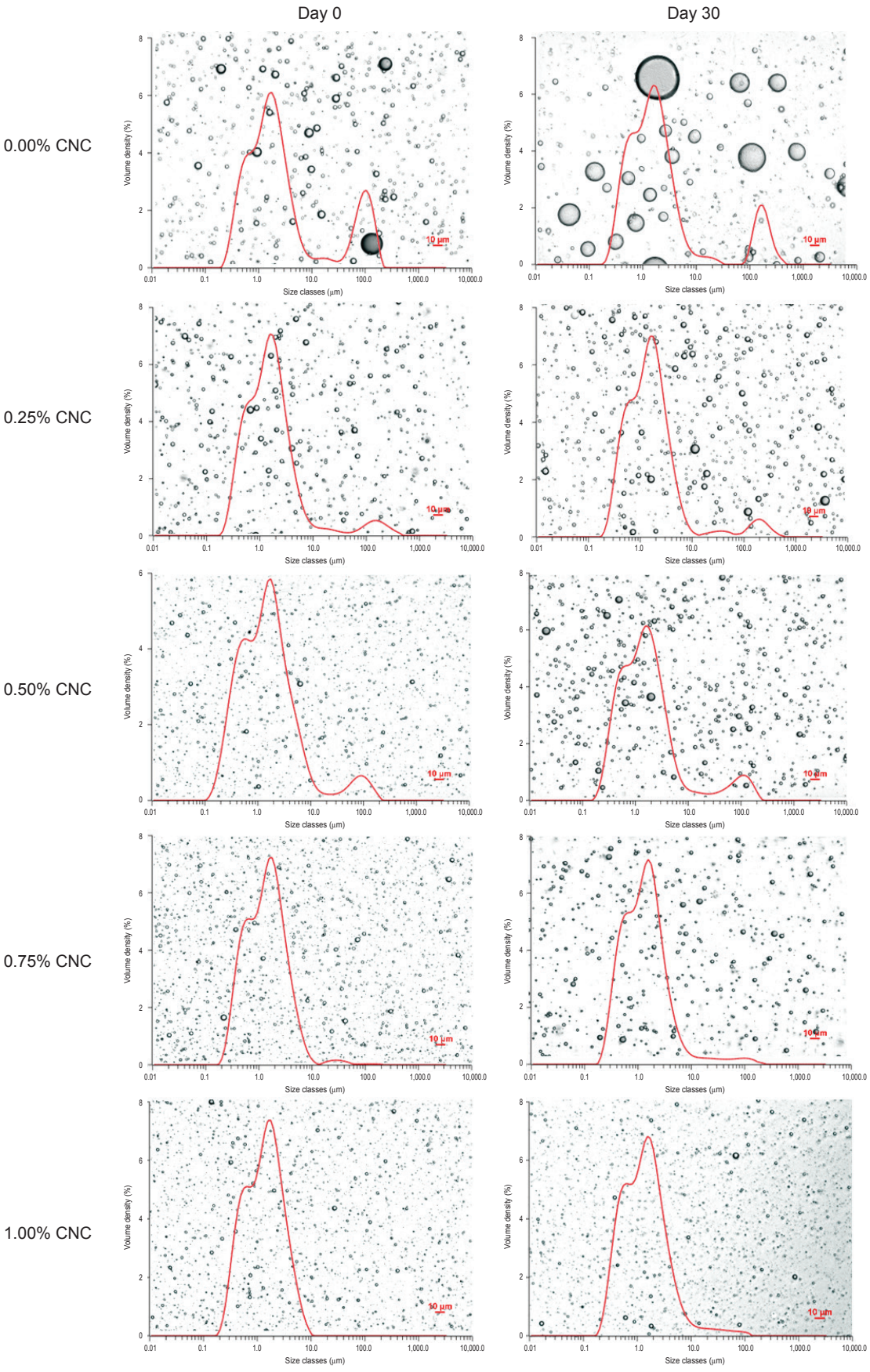

Figure 4. Optical micrographs of O/W emulsion (20/80, v/v) with the aqueous phase containing 1.00% (w/v) of SC and different CNC concentrations on day 0 and day 30. Droplet size distributions of respective emulsions determined by static light scattering are superimposed on the micrographs. The bar length represents 10 μm.

produce more free radicals (Chityala *et al*., 2016). All these possible mechanisms could be used to explain the slower rate for the formation of *p*-AV content as the secondary oxidation products when the rate of primary lipid oxidation got reduced.

Effect of CNC Content on FFA, TOTOX and Carotene Content

Oil acidity testing was carried out to determine the amount of FFA produced by the hydrolysis of oils during the storage period. It is well known that FFA, diacylglycerol (DAG) and monoacylglycerol (MAG) are made from the hydrolysis of oils and fats, and this acid value is commonly used to predict the oxidative stability of vegetable oil (Yodkaew *et al*., 2017). As depicted in *Figure 5c*, after five days of accelerated storage, the FFA value for 0.00% CNC and formulation contained 1.00% CNC increased from 0.0583 to 0.1820 and 0.0583 to 0.1300, respectively. The FFA value of 0.00% CNC was ~29% more than the formulation stabilised with a mixture of 1.00% CNC. A smaller FFA value gives better oil quality as most of the oil molecules remained as the original TAG arrangement without being degraded. The results showed that adding 1.00% CNC as the reinforced stabiliser could form a more stable emulsion against hydrolysis.

To further comprehend the rate of lipid oxidation, the TOTOX was calculated to estimate the oxidation deterioration of lipids. The TOTOX value is defined as a mathematical prediction of oxidative stability. It represents the sum of primary and secondary oxidation products, which correlates to oil deterioration (Pereira de Abreu *et al*., 2010). As depicted in *Figure 5d*, over the five days of accelerated testing, the TOTOX value for 0.00% CNC and formulation contained 1.00% CNC increased from 1.272 to 4.293 and 1.286 to 3.178, respectively. Formulation with a higher CNC concentration displayed a significantly lowered TOTOX value than the 0.00% CNC ($p<0.01$), suggesting that the presence of SC/CNC mixtures are effective in inhibiting lipid oxidation in emulsions. These results were similar to the previous oxidation tests (PV and *p*-AV), where the formulation contained 1.00% CNC exhibited the lowest rate of lipid oxidation compared with the other formulations. It was deduced that the lower the TOTOX value, the better the quality of the oil (Moigradean *et al*., 2012).

Carotenoids such as β-carotene serve as a biological antioxidant and a good source of vitamin A (Ferreira *et al*., 2016). The carotene originally found in red palm olein could be used as the first-line defence against lipid oxidation due to the available natural antioxidant. As depicted in *Figure 5e*, the amount of carotene available in red palm olein was approximately 460 mg kg^{-1} at day 0. The value decreased significantly ($p<0.05$) to 329 mg kg^{-1} for 0.00% CNC as compared with formulation contained 1.00% CNC which only decreased to 401 mg kg^{-1}. The visual observation for the red colour of the extracted palm oil obtained decreased, showing that the carotene content reduced over the storage period.

Overall, the results suggested that the emulsion stability against lipid oxidation increased in the following order: 0.00% CNC < 0.25% CNC < 0.50% CNC < 0.75% CNC < 1.00% CNC. This finding demonstrated where an increased concentration of polysaccharides enhanced overall emulsion stability and lower lipid oxidation rate. This is because formulations containing SC as an emulsion stabiliser were reported to form a stiff viscoelastic interfacial film with antioxidant activities such as free radical scavenging and reduced abilities (Cheetangdee and Benjakul, 2015). On the other hand, the addition of CNC into SC-based emulsions displayed better physical properties due to their highly crystalline nano and rigid rod-like structure, which could form a dense cellulose chain packed together with strong inter- and intramolecular hydrogen bonds (Grishkewich *et al*., 2017; Svagan *et al*., 2016) when used as a stabiliser in forming Pickering emulsion. Hence, SC reinforced with CNC as a food-grade stabiliser in forming Pickering emulsions could enhance oxidative stability. At the highest concentration of CNC (1.00%), the rate of lipid oxidation was much lower than other formulations.

Schematic representation of possible droplet stabilisation mechanisms in emulsions prepared using SC/CNC mixture is depicted in *Figure 6*. When the hydrophilic CNC was added as a secondary stabiliser into the hydrophobic SC, the resultant SC/CNC mixture with higher amphiphilic character could improve the wetting characteristics of the particles and facilitate effective partitioning at the O/W interface, thus, rendering the emulsions greater physical and oxidative stability (Yokota *et al*., 2019). It was postulated that the palm-olein based emulsions were stabilised by mixtures of SC and CNC through mixed possible processes of adsorption, complexation and layer-by-layer formation at the oil-water interface under the influence of the ultrasound field. It was also suggested that polysaccharides could be distributed on the surface of the droplets and the surrounding aqueous phase, thus, restricting the aggregation of oil droplets. Moreover, the anionic polysaccharide (contained surface hydroxyl and sulphate groups) with negatively surface charged could easily repel each other, which would keep the droplets further away from each other (Xu *et al*., 2017a). All these factors are essential in forming stable O/W Pickering emulsion as well as in lipid oxidation inhibition.

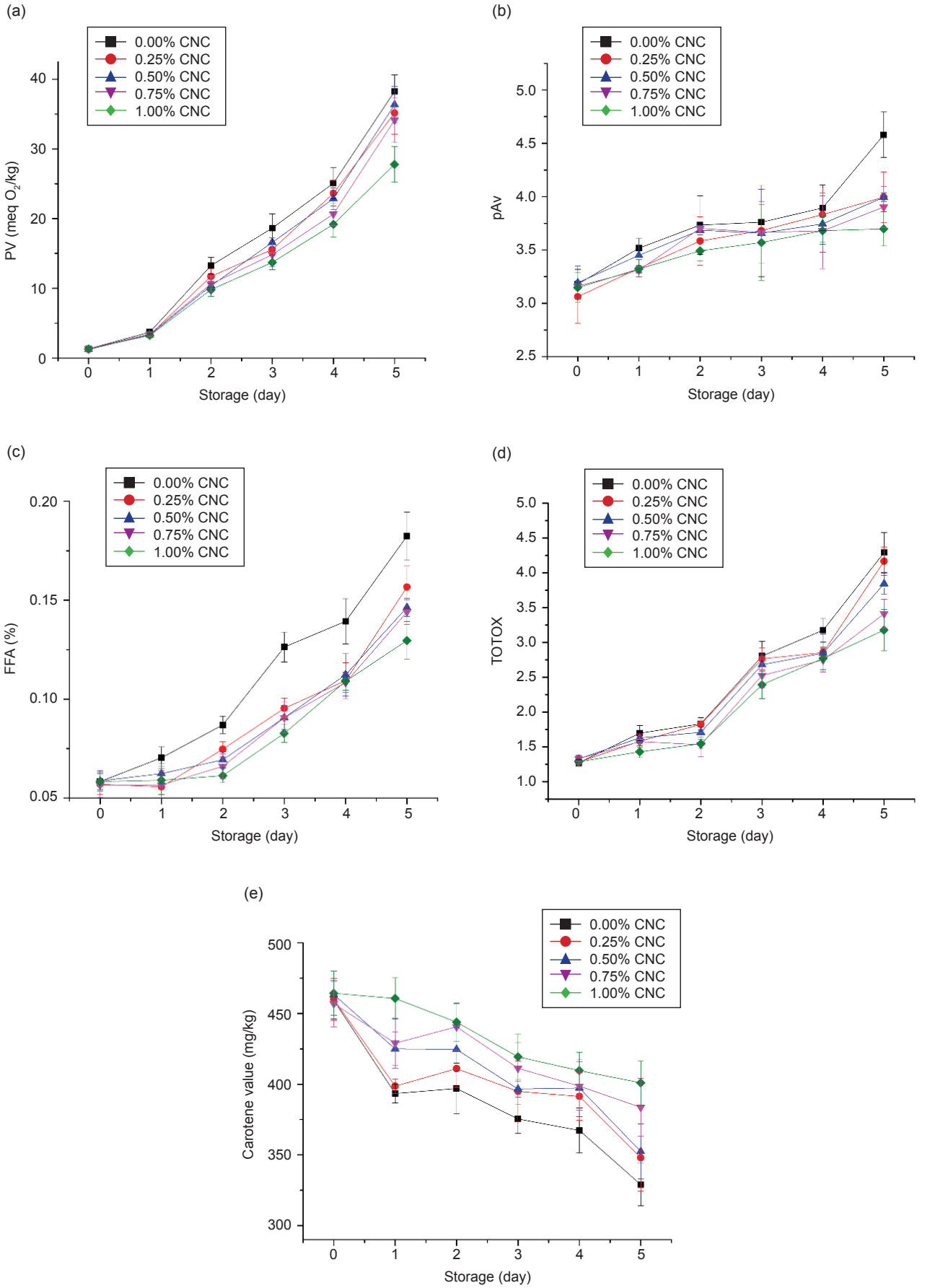

Figure 5. (a) Lipid peroxide value (PV), (b) p-anisidine value (p-AV), (c) free fatty acid (FFA) value, (d) total oxidation value (TOTOX), and (e) carotene value of O/W emulsion (20/80, v/v) with the aqueous phase containing 1.00% (w/v) of SC and different CNC concentrations as a function of accelerated testing at 90°C. Data represent means (n=3) with standard deviations (error bars).

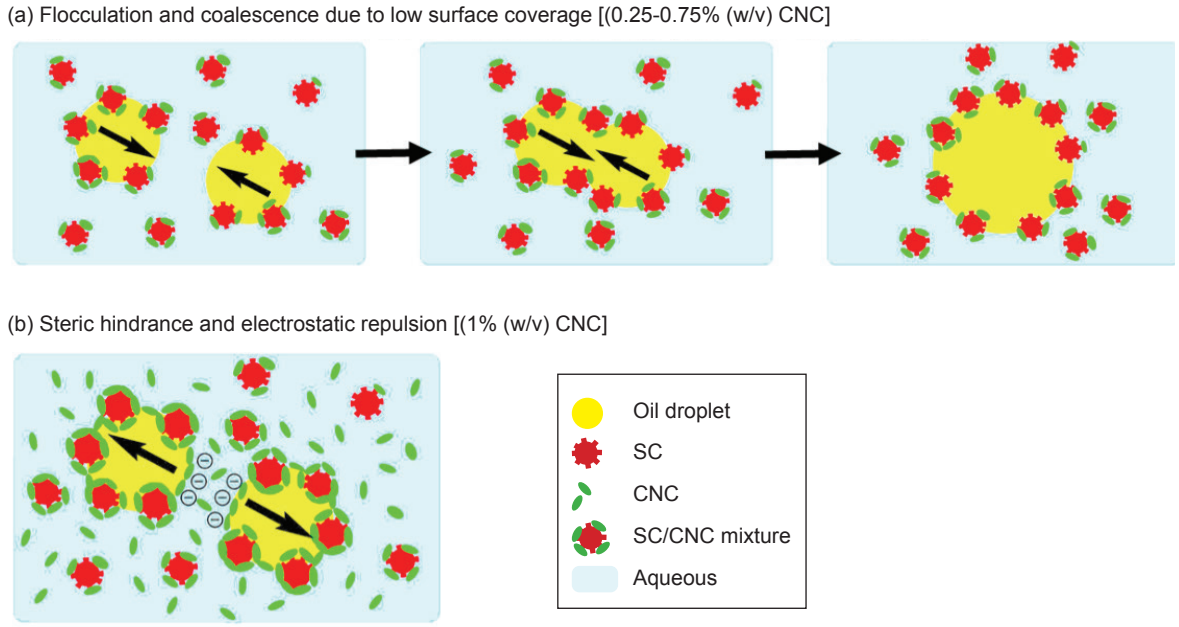

Figure 6. Schematic representation of proposed mechanism occurring (a) flocculation and coalescence, (b) steric hindrance and electrostatic repulsion of O/W emulsion (20/80, v/v) with the aqueous phase containing 1.00% (w/v) of SC and different CNC concentrations.

CONCLUSION

This study demonstrated the preparation of stable red palm O/W emulsions using a double biopolymer system containing SC and CNC particles. The effects of CNC concentration at a constant amount of SC on emulsion properties were investigated. The results showed that all the SC/CNC-stabilised emulsions exhibited improved physical stability, such as smaller droplet size, lower creaming index, higher surface charges, and improved oxidative stability compared to emulsions stabilised by SC alone. It was found that the mixed biopolymer-based emulsions containing 1.00% SC and 1.00% CNC were of superior stability against flocculation and coalescence during storage. The presence of negative charges around droplet surfaces also rendered the emulsions stable. Due to its antioxidant nature, the presence of CNC could inhibit the production of the first and second oxidation products of emulsions. Compared to SC-stabilised alone emulsions, SC/CNC-stabilised emulsions presented lower TOTOX value, FFA value with high carotene retention. Overall, our findings presented the beneficial effects of a dual biopolymer system comprising SC and CNC in improving the physical and oxidative stability of food-grade emulsions.

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REFERENCES

Ainie, K; Siew, W L; Tan, Y A; Noraini, I; Mohtar, Y; Tang, T S and Nuzulamri, I (2004). *MPOB Test Methods - A Compendium of Test on Palm Oil Products, Palm Kernel Products, Fatty Acids, Food Related Products and Others*. MPOB, Bangi. 414 pp.

American Oil Chemists' Society (AOCS) (1997). *Official Methods and Recommended Practices of the AOCS*. AOCS Press. Champaign, USA. 1200 pp.

Berton-Carabin, C C; Ropers, M H and Genot, C (2014). Lipid oxidation in oil-in-water emulsions: Involvement of the interfacial layer. *Compr. Rev. Food Sci. Food Saf.*, *13(5)*: 945-977.

Bonnie Tay, Y P and Choo, Y M (2000). Valuable minor constituents of commercial red palm olein: Carotenoids, vitamin E, ubiquinones and sterols. *J. Oil Palm Res.*, *12*: 14-24.

Cao, Y; Ai, N; True, A D and Xiong, Y L (2018). Effects of γ(−)-epigallocatechin-3-gallate γ incorporation on the physicochemical and oxidative stability of myofibrillar protein–soybean oil emulsions. *Food Chem.*, *245*: 439-445.

Chang, C and Nickerson, M T (2018). Encapsulation of omega 3-6-9 fatty acids-rich oils using protein-

based emulsions with spray drying. *J. Food Sci. Technol.*, *55(8)*: 2850-2861.

Cheetangdee, N and Benjakul, S (2015). Antioxidant activities of rice bran protein hydrolysates in bulk oil and oil-in-water emulsion. *J. Sci. Food Agric.*, *95(7)*: 1461-1468.

Chen, Q H; Zheng, J; Xu, Y T; Yin, S W; Liu, F and Tang, C H (2018). Surface modification improves fabrication of pickering high internal phase emulsions stabilised by cellulose nanocrystals. *Food Hydrocoll., 75*: 125-130.

Chew, C L; Ab Karim, N A; Quek, W P; Wong, S K; Lee, Y Y and Chan, E S (2021). Aerobic-liquor treatment improves the quality and deep-frying performance of refined palm oil. *Food Control, 126*: 108072.

Chityala, P K; Khouryieh, H; Williams, K and Conte, E (2016). Effect of xanthan/enzyme-modified guar gum mixtures on the stability of whey protein isolate stabilised fish oil-in-water emulsions. *Food Chem.*, *212*: 332-340.

Clegg, P S; Tavacoli, J W and Wilde, P J (2016). One-step production of multiple emulsions: Microfluidic, polymer-stabilised and particle-stabilised approaches. *Soft Matter, 12(4)*: 998-1008.

Pereira de Abreu, D A; Paseiro Losada, P; Maroto, J and Cruz, J M (2010). Evaluation of the effectiveness of a new active packaging film containing natural antioxidants (from barley husks) that retard lipid damage in frozen Atlantic salmon (*Salmo salar* L.). *Food Res. Int.*, *43(5)*: 1277-1282.

Ferreira, C D; da Conceição, E J L; Machado, B A S; Hermes, V S; de Oliveira Rios, A; Druzian, J I and Nunes, I L (2016). Physicochemical characterisation and oxidative stability of microencapsulated crude palm oil by spray drying. *Food Bioprocess Technol.*, *9(1)*: 124-136.

Francis, M J; Glover, Z J; Yu, Q; Povey, M J and Holmes, M J (2019). Acoustic characterisation of pH dependant reversible micellar casein aggregation. *Colloids Surf. A Physicochem. Eng. Asp.*, *568*: 259-265.

Grishkewich, N; Mohammed, N; Tang, J and Tam, K C (2017). Recent advances in the application of cellulose nanocrystals. *Curr. Opin. Colloid Interface Sci.*, *29*: 32-45.

Guzey, D and McClements, D J (2006). Formation, stability and properties of multilayer emulsions for application in the food industry. *Adv. Colloid Interface Sci.*, *128-130*: 227-248.

Ho, K W; Ooi, C W; Mwangi, W W; Leong, W F; Tey, B T and Chan, E S (2016). Comparison of self-aggregated chitosan particles prepared with and without ultrasonication pretreatment as Pickering emulsifier. *Food Hydrocoll.*, *52*: 827-837.

Hu, H Y; Xing, L J; Hu, Y Y; Qiao, C L; Wu, T; Zhou, G H and Zhang, W G (2016). Effects of regenerated cellulose on oil-in-water emulsions stabilised by sodium caseinate. *Food Hydrocoll.*, *52*: 38-46.

Jacobsen, C; Sørensen, A D and Nielsen, N (2013). Stabilisation of omega-3 oils and enriched foods using antioxidants. *Food Enrichment with Omega-3 Fatty Acids* (Jacobsen, C; Nielsen, N S; Horn, A F and Sørensen, A D M eds.). Woodhead Publishing. p. 130-149.

Jeon, T Y and Hong, J S (2014). Stabilisation of O/W emulsion with hydrophilic/hydrophobic clay particles. *Colloid Polym. Sci., 292(11)*: 2939-2947.

Le, T T; Saveyn, P; Hoa, H D and Van der Meeren, P (2008). Determination of heat-induced effects on the particle size distribution of casein micelles by dynamic light scattering and nanoparticle tracking analysis. *Int. Dairy J., 18(12)*: 1090-1096.

Li, K; Woo, M W; Patel, H and Selomulya, C (2017). Enhancing the stability of protein-polysaccharides emulsions via Maillard reaction for better oil encapsulation in spray-dried powders by pH adjustment. *Food Hydrocoll.*, *69*: 121-131.

Liu, H; Li, Y; Diao, X; Kong, B and Liu, Q (2018). Effect of porcine bone protein hydrolysates on the emulsifying and oxidative stability of oil-in-water emulsions. *Colloids Surf. A Physicochem. Eng. Asp.*, *538*: 757-764.

Liu, L; Zhao, Q; Liu, T; Kong, J; Long, Z and Zhao, M (2012). Sodium caseinate/carboxymethylcellulose interactions at oil-water interface: Relationship to emulsion stability. *Food Chem., 132(4)*: 1822-1829.

Lomova, M V; Sukhorukov, G B and Antipina, M N (2010). Antioxidant coating of micronsize droplets for prevention of lipid peroxidation in oil-in-water emulsion. *ACS Appl. Mater. Interfaces, 2(12)*: 3669-3676.

Low, L E; Siva, S P; Ho, Y K; Chan, E S and Tey, B T (2020). Recent advances of characterisation techniques for the formation, physical properties and stability of Pickering emulsion. *Adv. Colloid Interface Sci., 277*: 102117.

Low, L E; Tey, B T; Ong, B H; Chan, E S and Tang, S Y (2017). Palm olein-in-water Pickering

emulsion stabilised by Fe_3O_4-cellulose nanocrystal nanocomposites and their responses to pH. *Carbohydr. Polym.*, *155*: 391-399.

Low, L E; Wong, S K; Tang, S Y; Chew, C L; De Silva, H A; Lee, J M V; Hoo, C H and Kenrick, K (2019). Production of highly uniform Pickering emulsions by novel high-intensity ultrasonic tubular reactor (HUTR). *Ultrason. Sonochem.*, *54*: 121-128.

Luo, Y; Teng, Z; Wang, T T Y and Wang, Q (2013). Cellular uptake and transport of zein nanoparticles: Effects of sodium caseinate. *J. Agric. Food Chem.*, *61(31)*: 7621-7629.

Manoj, P; Fillery-Travis, A J; Watson, A D; Hibberd, D J and Robins, M M (1998). I. Creaming behavior. *J. Colloid Interface Sci.*, *207(2)*: 283-293.

McClements, D J (2015). *Food Emulsions: Principles, Practices and Techniques*. 3rd edition. CRC press. 714 pp.

Moigradean, D; Poiana, M A and Gogoasa, I (2012). Quality characteristics and oxidative stability of coconut oil during storage. *J. Agroaliment. Processes Technol.*, *18(4)*: 272-276.

Ortiz, D G; Pochat-Bohatier, C; Cambedouzou, J; Bechelany, M and Miele, P (2020). Current trends in Pickering emulsions: Particle morphology and applications. *Engineering, 6(4)*: 468-482.

Pinďáková, L; Kašpárková, V and Bordes, R (2019). Role of protein-cellulose nanocrystal interactions in the stabilisation of emulsion. *J. Colloid Interface Sci., 557*: 196-206.

Qiu, C; Zhao, M; Decker, E A and McClements, D J (2015). Influence of anionic dietary fibers (xanthan gum and pectin) on oxidative stability and lipid digestibility of wheat protein-stabilised fish oil-in-water emulsion. *Food Res. Int.*, *74*: 131-139.

Song, X; Zheng, F; Ma, F; Kang, H and Ren, H (2020). The physical and oxidative stabilities of Pickering emulsion stabilised by starch particle and small molecular surfactant. *Food Chem.*, *303*: 125391.

Sukyai, P; Anongjanya, P; Bunyahwuthakul, N; Kongsin, K; Harnkarnsujarit, N; Sukatta, U; Sothornvit, R and Chollakup, R (2018). Effect of cellulose nanocrystals from sugarcane bagasse on whey protein isolate-based films. *Food Res. Int.*, *107*: 528-535.

Svagan, A J; Koch, C B; Hedenqvist, M S; Nilsson, F; Glasser, G; Baluschev, S and Andersen, M L (2016). Liquid-core nanocellulose-shell capsules with tunable oxygen permeability. *Carbohydr. Polym.*, *136*: 292-299.

Wang, P; Chen, C; Guo, H; Zhang, H; Yang, Z and Ren, F (2018). Casein gel particles as novel soft Pickering stabilisers: The emulsifying property and packing behaviour at the oil-water interface. *Food Hydrocoll.*, *77*: 689-698.

Wong, S K; Supramaniam, J; Wong, T W; Soottitantawat, A; Ruktanonchai, U R; Tey, B T and Tang, S Y (2021). Synthesis of bio-inspired cellulose nanocrystals-soy protein isolate nanoconjugate for stabilisation of oil-in-water Pickering emulsions. *Carbohydr. Res.*, *504*: 108336.

Xu, X; Liu, W; Luo, L; Liu, C and McClements, D J (2017a). Influence of anionic polysaccharides on the physical and oxidative stability of hydrolyzed rice glutelin emulsions: Impact of polysaccharide type and pH. *Food Hydrocoll.*, *72*: 185-194.

Xu, L; Yu, X; Li, M; Chen, J and Wang, X (2017b). Monitoring oxidative stability and changes in key volatile compounds in edible oils during ambient storage through HS-SPME/GC–MS. *Int. J. Food Prop.*, *20(sup3)*: S2926-S2938.

Yodkaew, P; Chindapan, N and Devahastin, S (2017). Influences of superheated steam roasting and water activity control as oxidation mitigation methods on physicochemical properties, lipid oxidation and free fatty acids compositions of roasted rice. *J. Food Sci.*, *82(1)*: 69-79.

Yokota, S; Kamada, K; Sugiyama, A and Kondo, T (2019). Pickering emulsion stabilisation by using amphiphilic cellulose nanofibrils prepared by aqueous counter collision. *Carbohydr. Polym., 226*: 115293.

Zeng, T; Wu, Z l; Zhu, J Y; Yin, S W; Tang, C H; Wu, L Y and Yang, X Q (2017). Development of antioxidant Pickering high internal phase emulsions (HIPEs) stabilised by protein/polysaccharide hybrid particles as potential alternative for PHOs. *Food Chem.*, *231*: 122-130.

Zinoviadou, K G; Scholten, E; Moschakis, T and Biliaderis, C G (2012). Properties of emulsions stabilised by sodium caseinate–chitosan complexes. *Int. Dairy J.*, *26(1)*: 94-101.

Zoppe, J O; Venditti, R A and Rojas, O J (2012). Pickering emulsions stabilised by cellulose nanocrystals grafted with thermo-responsive polymer brushes. *J. Colloid Interface Sci.*, *369(1)*: 202-209.

SHORT COMMUNICATION

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SIMPLIFIED APPROACH FOR EARLY IDENTIFICATION OF SPONTANEOUS OIL PALM HAPLOID (*Elaeis guineensis*)

MUHAMMAD AZWAN, Z[1]; ZAKI, N M[1]*; NORDIANA, H M N[1]; MADON, M[2]; NORZIHA, A[1]; ZULKIFLI, Y[1]; CHAN, P L[1] and SINGH, R[1]

ABSTRACT

*Haploid technology facilitates the production of completely homozygous plants that are desirable in crop breeding. Having just one complete set of chromosomes in a haploid individual allows it to be doubled to produce a normal but pure 2n diploid plant. Here, we report a simple way to identify natural haploids of oil palm (*Elaeis guineensis *Jacq.) from screening 6400 abnormal germinated seeds. Initially, the germinated seeds were selected based on 12 unique 'off-type' morphological characteristics. The selected seeds were then grown and the seedlings were subjected to a second selection for three distinctive characteristics. Ploidy analysis with flow cytometry (FCM) and chromosome karyotyping confirmed the haploidy of one seedling with stunted height and size. Further analysis with the True-to-Type single nucleotide polymorphism (SNP) panel demonstrated that the plant was homozygous at all the loci tested, confirming its haploid status. This study has established a simple and systematic strategy that assists in accelerating early identification of oil palm spontaneous haploid.*

Keywords: abnormal germinated seed, *Elaeis guineensis*, haploid.

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INTRODUCTION

Haploids are a valuable source in crop improvement (Germana *et al.*, 2011), widely used by doubling up to produce homozygous diploids rapidly. This increases selection efficiency in the F1 generation and overcomes inbreeding depression, all in all, to shorten the breeding cycle in conventional breeding (Dwivedi *et al.*, 2015). Doubled haploid breeding is becoming an important tool in many programs, such as in brassicas (Xu *et al.*, 2007) and maize (Chaikam *et al.*, 2019). Due to the complete homozygosity of doubled haploid lines, the technology is also highly desirable for molecular and gene mapping research (Chaikam *et al.*, 2019).

The occurrence of spontaneous haploids that are naturally derived without any manipulation is frequently discovered in small frequency (Chase *et al.*, 1969; Dunwell *et al.*, 2010). The first natural haploid was a weed reported by Dorothy Bergner (Blakeslee, 1922), then in tobacco, peach and wheat (Forster *et al.*, 2007; Kostoff, 1929; Yahata *et al.*, 2005). In oil palm, about 0.02% spontaneous haploids were identified from 60 millions abnormal seedlings (Dunwell *et al.*, 2010).

Various techniques have been applied to detect haploids. A conventional yet economical approach is by screening their phenotypic features, which reports indicate are stunted and having off-type characteristics due to the chromosome aberration (Yahata *et al.*, 2005). Another approach, chromosome counting, is also conventional and reliable. The haploid has only a half set of chromosomes. In

[1] Malaysian Palm Oil Board,
6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.

[2] 9, Jalan BM 5/12, Bandar Bukit Mahkota,
43000 Kajang, Selangor, Malaysia.

* *Corresponding author email: hariza@mpob.gov.my*

E. guineensis, chromosome counting was established by Madon *et al.* (1995), who found 2n=32 chromosomes categorised into three groups – one long, eight medium and seven short pairs, with the chromosomes sizes ranging from 1.00-3.89 μm. Other than these conventional approaches, flow cytometry (FCM), a technique used to detect and measure physical and chemical characteristics of cells has become the method of choice to measure the deoxyribonucleic acid (DNA) content and ploidy level in plants (Dolezel *et al.*, 2007). For this reason, FCM is a reliable and fast method to identify haploid plants and has been manipulated as a high-throughput method to screen numerous plant species with various ploidy levels (Nasution *et al.*, 2013; Ochatt, 2008).

The oil palm (*Elaeis guineensis* Jacq.) is a productive and versatile oil crop with a diploid genome. This perennial produces 36.5% of the total world vegetable oils - 75.5 million tonnes in 2019, even higher than soybean, the previous major producer (MPOB, 2019). The demand for palm oil for both edible and non-edible uses is expected to increase with the growing world population. However, improving the crop is difficult due to its long reproductive cycle (10-15 years per breeding generation) and sheer size, requiring huge areas from the breeding trials (Seng *et al.*, 2016). Hence, to overcome such hurdles, biotechnology means or other related techniques that can boost advanced breeding efficiency should be applied. Thus, in this study, a reliable and straightforward approach is proposed for the medium-scale screening for oil palm haploids at the nursery stage to facilitate oil palm breeding.

MATERIALS AND METHODS

Plant Materials

A total of 6400 abnormal germinated seeds from advanced breeding materials and germplasm were obtained from crosses of DxD, DxP, (OxP)xP and TxT (D: *dura*; P: *pisifera*; O: *oleifera* T: *tenera* fruit form) at MPOB Kluang Research Station, Johor, Malaysia.

Morphological Screening of Natural Haploids

Identification of naturally occurring haploid was initiated with seed morphology screening. Two phases of morphological screening on abnormal germinated seeds/seedlings were performed in this study. The first screening was performed according to Wan Ibrahim *et al.* (2011) based on 12 'off-type' morphological characteristics of germinated seeds. The seed characteristics involved in this screening are described in *Figure 1*. The classified abnormal germinated oil palm seeds were then planted according to their abnormal categories. This was followed by the second morphological screening on the three-month-old seedlings based on three characteristics: stunted growth (small size of leaves and height), grassy (narrow and erect grass-like leaves) and, rolled (coiled leaves and twisted stem) leaves (*Figure 2*). Any normal seedlings (typically with 3-4 dark green leaves) were discarded. Ploidy analysis of the abnormal seedlings was carried out with a FACSCalibur™ flow cytometer (Becton Dickinson, USA).

Ploidy Level Analysis

For nuclei preparation, the sampling and processing of leaf samples were performed as described by Madon *et al.* (2005) with slight modification. Three replicates of each different parts of frond +1 (base, middle and top) were sampled. Each piece (1.0 cm x 1.0 cm) of leaf was cut to fine slices and incubated in 1.0 mL lysis buffer (LBO1) containing 15 mM ethylenediaminetetraacetic acid, disodium salt (Na_2EDTA), 80 mM potassium chloride (KCl), 20 mM sodium chloride (NaCl), 0.5 mM spermine, 15 mM β-mercaptoethanol, 0.15 Triton X-100 (pH 7.5), 50 mg propidium iodide (PI) fluorescent stain and 50 μg RNaseA. The diploid oil palm was used as the standard control. The fluorescent intensity of the nuclei was measured using an argon ion laser at 488 nm in a FACSCalibur™ flow cytometer (Becton Dickinson, USA). For each sample, the 2C DNA histogram peak obtained from the CellQuest software was compared with 2C DNA content of the standard control.

Chromosome Counting

The root tips from the germinated seedlings were collected for chromosome preparation. The fresh tips were pre-treated with 2 mM 8-hydroxyquinoline for 5-6 hr and fixed in 3:1 absolute ethanol: glacial acetic acid based on Madon (2000). The roots were then rinsed three times with enzyme buffer (0.01 M citric acid-sodium citrate, pH 4.6). The root cap was removed using a pair of fine forceps under a dissecting microscope (Meiji Stereo). About 1 cm terminal region from the tip (which included the meristem) was incubated in enzyme mix (2% cellulose and 20% pectinase) at 37°C for 1-3 hr, placed on a glass slide with a drop of 60% acetic acid, squashed and the debris was removed leaving only a cell suspension. The suspension was covered with a glass cover slip, then tapped a few times and thumb pressure applied to spread out the chromosomes. Next, the cover slip was sealed using nail polish to avoid the specimen from drying. The slide was viewed under 40x magnification of microscope (Carl Zeiss Axioplan, Germany).

Figure 1. Morphologies of normal and abnormal oil palm germinated seeds. (a) normal oil palm germinated seeds, (b) stunted plumule, (c) stunted plumule and radicle, (d) stunted radicle, (e) thin radicle, (f) plumule and radicle in 90° position, (g) branched radicle, (h) branched plumule, (i) ratio of plumule and radicle exceed 1:1, (j) twin seedlings (V-shaped), (k) doubleton with stunted plumule/ radicle (seedling has two stunted radicles and plumules), (l) abnormal colour plumule, brownish and (m) abnormal colour radicle.

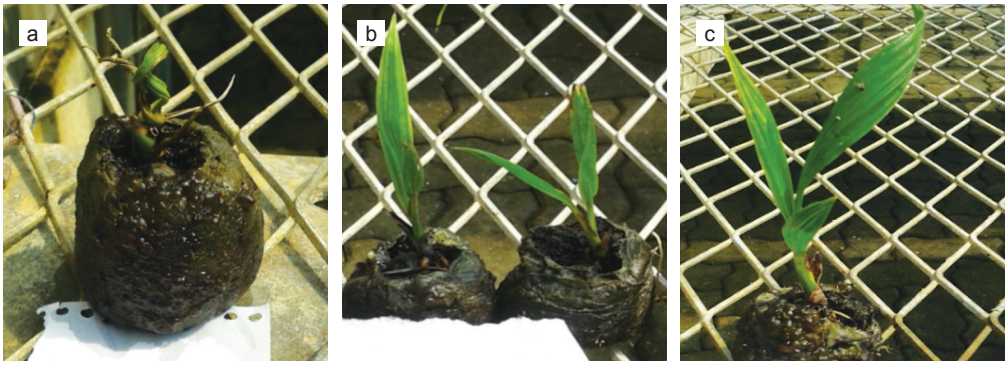


Figure 2. Morphologies of oil palm abnormal seedlings in second stage screening. (a) stunted, (b) grassy leaves, and (c) rolled leaves.

DNA Extraction

Two grams of spear leaves from the haploid plant was ground to a fine powder in liquid nitrogen using mortar and pestle. Genomic DNA was extracted using the modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990; Suzana *et al.*, 2015). The quality of the DNA was evaluated as described by Rahimah *et al.* (2006).

Genotyping using SureSawit™ True-to-Type Single Nucleotide Polymorphism (SNP) Panel

DNA from the haploid and 15 individual diploid *dura* x *pisifera* (DxP) palms were genotyped using SureSawit™ True-to-Type SNP panel following the method of Ooi *et al.* (2019).

RESULTS

Of the 6400 abnormal germinated seeds examined, the preliminary screening identified 2495 (39%) with 12 'off-type' morphological characteristics (*Figure 1*). The subsequent morphological screening of the three-month-old seedlings derived from the 2495 seeds has classified a total of 1346 seedlings with three prominent morphological traits; grassy leaf (673), stunted in growth (520) and seedling with rolled leaves (153). The remaining 1149 possessed other phenotypic features and were discarded.

The 1346 abnormal seedlings identified based on three characteristics (stunted, grassy, rolled leaves) were further analysed for their ploidy level using FCM. Of these, only one haploid with morphology of stunted in both height and size (*Figure 3a*) was found. The fluorescence intensity peak of sole haploid was observed at channel 130-160 (*Figure 3d*). Chromosome counting confirmed the palm has only 16 chromosomes in the palm (*Figure 3e*). Further genotyping with True-to-Type SNP panel showed 100% homozygous SNP for all its loci tested compared to those of the 15 individual DxP palms. The DxP palms exhibited SNP homozygosity of less than 60% (*Figure 4*).

DISCUSSION

The occurrence of spontaneous haploids is very low. In most studies, haploids are produced by chemical treatment or induced method (Melchinger *et al.*, 2019). It is still not clear how haploids are spontaneously produced. Conceptually, there are several ways, namely spontaneous polyembryony, parthenogenesis and androgenesis (Horlow and Raquin, 1998; Mishra and Gowswami, 2014). Uniparental chromosome elimination as shown in *Arabidopsis thaliana* (Ravi and Chan, 2010) is another means leading to haploidy, although few studies demonstrated that this occurred in interspecific hybrids (Liu *et al.*, 2014; Wedzony *et al.*, 2009).

Numerous studies have associated abnormal germinated seeds, or 'off types', with haploid formation (Garavello *et al.*, 2019). The actual 'off-type' morphologies observed in germinated seedlings that may be linked to haploid plants have never been described clearly for oil palm. Therefore, a systematic screening for haploids was carried out on a massive pool of abnormal germinated oil palm seeds based on unique morphological characteristics. Such a simple approach, if successful, would be a practical way to obtain haploid oil palm on a medium scale. Moreover, the identification of the exclusive characteristic of the haploid seedlings with additional phenotypic screening may form an ideal and robust approach in identifying haploid individuals. Large numbers of spontaneous haploids and doubled haploids in oil palm were only reported by Dunwell *et al.* (2010). In our study, the abnormalities such as stunted radicle and plumule were observed during the germination stage prior to the development of the seeds into three-month-old seedlings with stunted height and size compared to the normal palm. This growth habit is similar to that of haploids in other plants (Aleza *et al.*, 2009; Maluszynska *et al.*, 2003).

In addition to morphology screening, several other methods, such as FCM, chromosome counting and SureSawit™ True-to-Type SNP panel were used to ascertain haploid status. FCM is fast and convenient (Dolezel *et al.*, 2007; Sliwinska, 2018). It robustly determined the ploidy level of the putative haploid in numerous plant research involving induction of haploids and doubled haploids (Garavello *et al.*, 2019; Gu *et al.*, 2013). From the FCM result, the ploidy level was determined by comparing the known ploidy (represented as histogram peak) of a reference plant genome to the unknown species. The 2C DNA histogram peak for a reference diploid oil palm is between 260-320 (Madon *et al.*, 2008). In comparison to the oil palm diploid, a single 2C DNA histogram peak at the 130-160 channel was detected for the sole haploid palm found (*Figure 3d*). The classical chromosome counting was also performed to confirm the chromosome number of this haploid. Although cytological analysis is time-consuming, it is, nevertheless, the most reliable approach to confirm the haploidy of any species as the chromosomes are easily counted in mitotic cells obtained from the root tip (Madon *et al.*, 1998; Zaki *et al.*, 2017). Chromosome counting showed that the candidate haploid palm has 16 chromosomes, half the number of chromosomes in the diploid palm. Furthermore, results from SNP analysis also confirmed the homozygosity of the haploid palm, with only a single allele detected across all the 135 loci tested. With its bi-allelic nature, SNP markers are

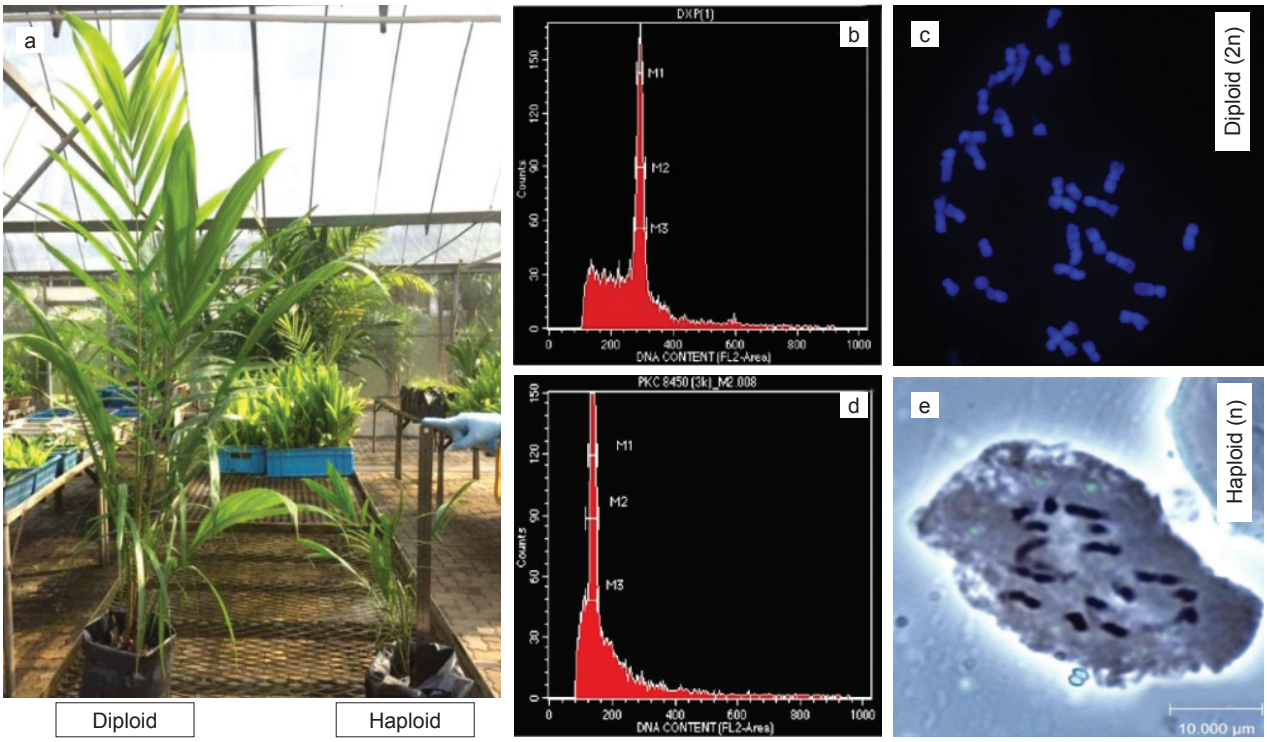
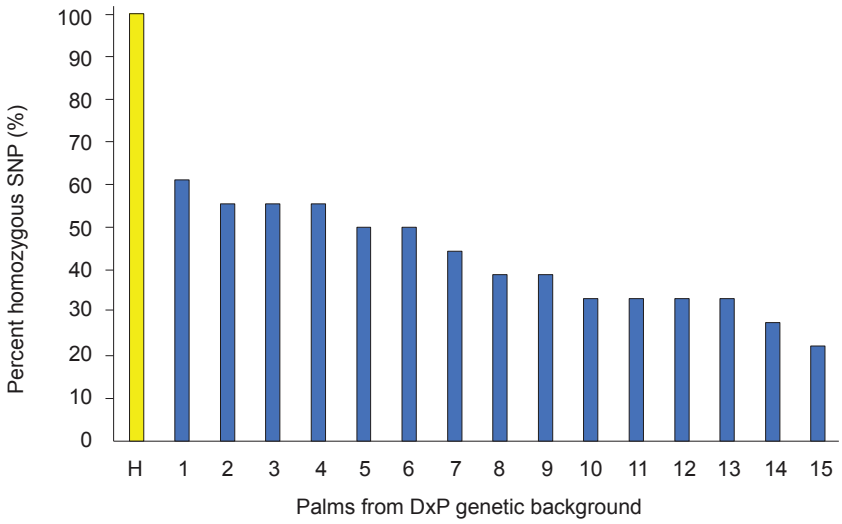


Figure 3. Comparative features of diploid and haploid oil palm. (a) morphology of normal diploid (left) and stunted haploid (right) oil palm; (b) fluorescent intensity histogram peak of diploid at 260-320 channel; (c) mitotic chromosome preparation showing 32 diploid chromosomes [stained with 4',6-diamidino-2-phenylindole (DAPI)] (bar=10 µm) (d) fluorescent intensity histogram peak of haploid at channel 130-160 in FCM analysis; (e) mitotic chromosome preparation showing 16 haploid chromosomes (bar=10 µm).



Note: SNP - single nucleotide polymorphism.

Figure 4. Genotyping of haploid palm (H) and 15 DxP palms (1-15) using True-to-Type SNP panel.

easier to use than microsatellite markers as reported by Mammadov *et al.* (2012).

This article provides a simple, systematic and robust approach for identification of spontaneous oil palm haploids. As an alternative approach to Dunwell *et al.* (2010), this study established three steps screening at smaller scale, comprising of morphological screening of germinated seeds and seedlings with the potential exclusive characteristics, verification of ploidy level with FCM, and cytological analysis to validate the haploid chromosome number.

Moreover, the substitution of massive simple sequence repeat (SSR) screening to True-to-Type SNP panel in determining the homozygosity of haploid candidate is another beneficial alternative in simplifying the spontaneous haploid screening process as a whole.

CONCLUSION

This study provides information on the identification of natural haploids in oil palm. Improvement in the approach is critical for haploid production in oil palm. FCM, chromosome counting and SNP analysis used here, can be applied as haploid detection tools in oil palm. Early detection of haploids at seed germination stage will benefit oil palm breeders.

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REFERENCES

Aleza, P; Juárez, J; Hernández, M; Pina, J A; Ollitrault, P and Navarro, L (2009). Recovery and characterization of a *Citrus clementina* Hort. ex Tan. 'Clemenules' haploid plant selected to establish the reference whole genome citrus. *BMC Plant Biol., 9*: 110.

Blakeslee, A F (1922). A haploid mutant in the Jimson weed, *Datura stramonium. Science, 55*: 646-647.

Chaikam, V; Molenaar, W; Melchinger, A E and Boddupalli, P M (2019). Doubled haploid technology for line development in maize: Technical advances and prospects. *Theor. Appl. Genet., 132*: 3227-3243.

Chase, S S (1969). Monoploids and monoploid-derivatives of maize (*Zea mays* L.). *Bot. Rev., 35*: 117-167.

Dunwell, J M; Wilkinson, M J; Nelson, S; Wening, S; Sitorus, A C; Mienanti, D; Alfiko, Y; Croxford, A D; Ford, C S; Forster, B P and Caligari, P D S (2010). Production of haploids and doubled haploids in oil palm. *BMC Plant Biol., 10*: 218.

Dolezel, J; Greilhuber, J and Suda, J (2007). Estimation of nuclear DNA content in plants using flow cytometry. *Nat. Protoc., 2*: 2233-2244.

Doyle, J J and Doyle, J L (1990). Isolation of plant DNA from fresh tissue. *Focus, 12*: 13-15.

Dwivedi, S L; Britt, A B; Tripathi, L; Sharma, S; Upadhyaya, H D and Ortiz, R (2015). Haploids: Constraints and opportunities in plant breeding. *Biotechnol. Adv., 33*: 812-829.

Garavello, M; Cuenca, J; Dreissig, S; Fuchs, J; Houben, A and Aleza, P (2019). Assessing ploidy level analysis and single pollen genotyping of diploid and euploid citrus genotypes by fluorescence-activated cell sorting and whole-genome amplification. *Front. Plant Sci., 10*: 1-12.

Germana, M A (2011). Gametic embryogenesis and haploid technology as valuable support to plant breeding. *Plant Cell Rep., 30*: 839-857.

Gu, C; Liu, Q Z; Yang, Y N; Zhang, S J; Khan, M A; Wu, J and Zhang, S L (2013). Inheritance of hetero-diploid pollen S-haplotype in self-compatible tetraploid Chinese cherry (*Prunus pseudocerasus Lindl*). *PLoS ONE, 8(4)*: e61219.

Horlow, C and Raquin, C (1998). A critical analysis of existing haploidization techniques. *Androgenesis and Haploid Plants* (Chupeau, Y; Caboche, M and Henry, Y eds.). Springer, Berlin Heidelberg New York. p. 7-23.

Kostoff, D (1929). An androgenic Nicotiana haploid. *Zeitschrift für Zellforschung und Mikroskopische Anatomie, 9*: 640-642.

Liu, D; Zhang, H; Zhang, L; Yuan, Z; Hao, M and Zheng, Y (2014). Distant hybridization: A tool for interspecific manipulation of chromosomes. *Alien Gene Transfer in Crop Plants* (Pratap, A and Kumar, J eds.). Vol. 1: Innovations, Methods and Risk Assessment. Springer. p. 25-42.

Madon, M (2000). Cytogenetical mapping of oil palm chromosomes. Ph.D thesis, Universiti Kebangsaan Malaysia.

Madon, M; Clyde, M M and Cheah, S C (1995). Cytological analysis of *Elaeis guineensis* (*tenera*) chromosomes. *Elaeis, 7*: 122-134.

Madon, M; Clyde, M M and Cheah, S C (1998). Cytological analysis of *Elaeis guine*ensis and *Elaeis oleifera* chromosomes. *J. Oil Palm Res., 10(1)*: 68-91.

Madon, M; Clyde, M M; Hashim, H; Mohd Yusuf, Y; Mat, H and Saratha, S (2005). Polyploidy induction of oil palm through colchicine and oryzalin treatments. *J. Oil Palm Res., 17*: 110-123.

Madon, M; Phoon, L Q; Clyde, M M and Mohd Din, A (2008). Application of flow cytometry for estimation of nuclear DNA content in *Elaeis*. *J. Oil Palm Res., 20*: 447-452.

Maluszynska, M; Kasha, K J; Forster, B P and Szarejko, I (2003). Published protocols for other crop plant species. *Doubled Haploid Production in*

Crop Plants: A Manual (Maluszynski, M; Kasha, K J; Forster, B P and Szarejko, I eds.). Dordrecht, Kluwer. p. 309-335.

Mammadov, J; Aggarwal, R; Buyyarapu, R and Kumpatla, S (2012). SNP markers and their impact on plant breeding. *Int. J. Plant Genomics, 2012*: 1-11.

Melchinger, A E; Schipprack, W; Wurschum, T; Chen, S and Technow, F (2019). Rapid and accurate identification of *in vivo*-induced haploid seeds based on oil content in maize. *Sci. Rep., 3*: 21-29.

Mishra, V K and Gowswami, R (2014). Haploid production in higher plant. *Int. J. Chem. Biol. Sci., 1*: 26-45.

MPOB (2019). *Malaysian Oil Palm Statistics 2019*. 39th edition. MPOB, Bangi. p. 185.

Nasution, O; Sitorus, A C; Nelson, S P C; Forster, B P and Caligari, P D S (2013). A high-throughput flow cytometry method for ploidy determination in oil palm. *J. Oil Palm Res., 25(2)*: 265-271.

Ochatt, S J (2008). Flow cytometry in plant breeding. Cytometry A. *PubMed, 73(7)*: 581-598.

Ooi, LC-L; Low, E-T L; Ordway, J; Marjuni, M; Yaakub, Z; Jiang, N; Smith, S; Bacher, B; Garner, P A; Leinenger, M T; Sander, N; Chan, P L; Ong, P W; Ong-Abdullah, M; Nookiah, R; Abd Manaf, M A; Lakey, N; Sambanthamurthi, R and Singh, R (2019). SureSawit™ True-to-Type-A high throughput universal single nucleotide polymorphism panel for DNA fingerprinting, purity testing and origin verification in oil palm. *J. Oil Palm Res., 31(4):* 561-571.

Rahimah, A B; Cheah, S C and Rajinder, S (2006). Freeze-drying of oil palm (*Elaeis guineensis*) leaf and its effect on the quality of extractable DNA. *J. Oil Palm Res., 18*: 296-304.

Ravi, M and Chan, S W (2010). Haploid plants produced by centromere mediated genome elimination. *Nature, 464*: 615-618.

Seng, T; Ritter, E; Mohamed Saad, S H; Leao, L-J; Singh, R; Faridah, Q Z; Tan, S-G; Syed Alwee, S S R and Roa, V (2016). QTLs for oil yield components in an elite oil palm (*Elaeis guineensis*) cross. *Euphytica, 212*: 399-425.

Sliwinska, E (2018). Flow cytometry - A modern method for exploring genome size and nuclear DNA synthesis in horticultural and medicinal plant species. *Folia Hortic., 30*: 103-128.

Suzana, M; Rahimah, A R; Maizura, I and Singh, R (2015). A simple and rapid protocol for isolation of genomic DNA from oil palm leaf tissue. *J. Oil Palm Res., 27*: 282-287.

Wan Ibrahim, W H; Zulkifli, Y; Norziha, A; Rosimah, N and Madon, M (2011). Obtaining oil palm haploids via screening for naturally occurring haploid (NOH) in oil palm germinated seeds and wide hybridization. *Proc. of the PIPOC International Palm Oil Congress 2011*. MPOB, Bangi.

Wędzony, M; Forster, B P; Żur I; Golemiec, E; Szechyńska-Hebda, M; Dubas, E and Gotebiowska, G (2009). Progress in doubled haploid technology in higher plants. *Advances in Haploid Production in Higher Plants* (Touraev, A; Forster, B P and Jain, S M eds.). Springer, Dordrecht. p. 1-33.

Xu, L; Najeeb, U; Tang, G X; Gu, H H; Zhang, G Q; He, Y and Zhou, W J (2007). Haploid and doubled haploid technology. *Adv. Bot. Res., 45*: 181-216.

Yahata, M; Kurogi, H; Kunitake, H; Nagano, K; Yabuya, T; Yamashita, K and Komatsu, H (2005). Evaluation of reproductive functions in a haploid pummelo by crossing with several diploid citrus cultivars. *J. Jpn. Soc. Hortic. Sci., 74*: 281-288.

Zaki, N M; Singh, R; Nordiana, H M N; Muhammad Azwan, Z; Smith, S W; Schwarzacher, T; Madon, M and Heslop Harrison, J S (2017). Short Communication: Towards development of *Elaeis guineensis* chromosome - arm specific markers and their utility across the *Elaeis* genus. *J. Oil Palm Res., 29(4)*: 594-599.

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