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Original Article

Features of *Jatropha curcas* seed oil in relation to different pollination methods

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Abstract

Physic nut (*Jatropha curcas* L.) is an attractive feedstock for producing standard-satisfied biodiesel. This study aimed to fill a knowledge gap of some aspects on fruit and seed biology of this biodiesel plant, by determining whether or not the pollination method affected seed oil accumulation and the development of fruit, embryo and endosperm as well as lipid storage in embryo and endosperm. For seed oil content, no significant difference was observed among open, cross-, self- and non-pollinated (apomixis) seeds. The maximum fruit and seed set, and the highest content of oleic acid were obtained from open pollination. The fruit diameter growth was well fit by a single sigmoid shape ($R^2 = 0.90$). The contents of oil and oleic acid were higher in the endosperm than in the embryo. The oil bodies isolated from both embryo and endosperm tissues mainly contained triacylglycerol (TAG) and integrated proteins termed caleosins and oleosins.

Keywords: pollination method, oil content, oleic acid, oil body, *Jatropha* seed

1. Introduction

The physic nut is a promising alternative source of renewable biodiesel because its inedible oil and fatty acid composition is analogous to that of fossil fuel (Mazumdar, Borugadda, Goud, & Sahoo, 2012). A previous study showed that this species could produce fruit via three reproductive modes: cross-, self- and non-pollination (apomixis) (Kaur, Dhillon, & Gill, 2011). However, there is no available report about the effects of pollination method on oil features of physic nut grown locally in Thailand. Thus, an association between pollination route and seed lipid profiles are needed for this plant – for applying the best pollination that would give the best productivity in the plantation.

Developments of fruit, seed and embryo were examined in this species grown in Mexico (Catzín-Yupit, Ramírez-Morillo, Pool, & Loyola-Vargas, 2014). Nevertheless, the correlation among fruit diameter, embryo development, endosperm ontogeny and period after pollination remains poorly known for this plant. This relationship would be helpful

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for a breeding program, such as for choosing seeds with desired-stage endosperm for *in vitro* triploid plant culture, essentially for developing new plant varieties (Wang, Cheng, Zhi, & Xu, 2016). In addition, those insights could be important for crop management to improve yield and quality (Wubs, Ma, Heuvelink, Hemerik, & Marcelis, 2012).

The seed storage lipid is mostly in a form of TAG preserved in a special organelle called oil body, which is regularly enclosed by phospholipid monolayer rooted by proteins named oleosin, caleosin and steroleosin (Tzen, Cao, Laurent, Ratnayake, & Huang, 1993). Both embryo and endosperm could serve as seed oil storage sites but with different contents and fatty acid compositions (Dussert *et al*., 2013; Errouane *et al*., 2015). Nevertheless, very little is provided in the literature for the lipid characterization between embryo and endosperm of physic nut seed. Oil droplets could be noticed in the embryo of this species (Reale *et al*., 2012); however, the oil content and fatty acid components in this tissue have never been determined. Thus, improved knowledge on lipid contribution in the seed tissues is a prerequisite for further examination of mechanisms directing fluctuations in lipid composition, in order to improve the oil productivity.

Consequently, this research has attempted to study 1) the effects of pollination route on seed oil accumulation, and 2) the development of fruit, embryo and endosperm as well as lipid storage in the embryo and endosperm obtained with open pollination.

2. Materials and Methods

2.1 Plant materials

This investigation was carried out during 2013-2015 by employing local *J. curcas* trees grown at Trang Agricultural Occupation Promotion and Development Center, Trang province, Thailand.

2.2 Manipulation of open, cross-, self- and non-pollination

For open pollination, approximately 300-500 female flowers from around 10 sampling inflorescences per tree (10 healthy plants) were tagged in the morning of anthesis day and left for free pollination. The date in which the flowers were marked was designated as pollination day. For cross-, self- and non-pollination, the entire male flowers were removed before the opening of female flowers in the same inflorescence and then the remaining female flowers were covered with paper bags. For cross-pollination, the viable pollens from different trees were transferred to the responsive stigmata, while the viable pollens from other inflorescences in the same individual plant were moved to the receptive stigmata in self-pollination. For non-pollination, the emasculated flowers were bagged without pollination. The samples were continuously covered by the paper bags for 10 days. Percentages of fruit set and number of seeds per fruit were computed against 120 initially labeled female flowers and from 45 fruits, respectively.

2.3 Development of fruit, embryo and endosperm

For growth observation, equatorial diameters of 24 sampled fruits were measured after pollination at 3-day intervals up to 48 days after pollination (DAP). To examine embryo and endosperm development, the fruits collected at every 3 DAP were fixed in FAA II (formaldehyde: glacial acetic acid: 70% ethyl alcohol, 5: 5: 90 v/v/v) for 48 h, dehydrated with a tertiary-butyl-alcohol series and embedded in Paraplast Plus. Paraffin sections of 6 µm were stained with hematoxylin and safranin (Ruzin, 1999). The presence of lipid droplets in mature embryo and endosperm was detected by staining the fresh cut samples (20 µm thick) with Oil Red O (Brown, 1969). Samples were visualized with an Olympus-BX51 light microscope equipped with a DP-72 digital camera (Olympus Optical Co. Ltd., Japan).

2.4 Analysis of oil content and fatty acid composition

The samples (1 g dry weight) of crushed seeds from all pollinations, and the embryo and endosperm from open pollination were extracted using a Soxhlet extractor with *n*-hexane for 6 h. Oil content, as a percentage of dry weight, was calculated from 3 replicates. The oil was hydrolyzed in KOH/MeOH, mixed with HCl/MeOH and extracted using petroleum ether (Jham, Teles, & Campos, 1982) before subjecting to biodiesel analysis. The fatty acid methyl ester (FAME) analysis was accomplished using gas chromatography (6890, Aligent, USA) fitted with a flame ionization detector and a capillary column selected for biodiesel FAME (30 m × 0.32 mm × 0.25 µm). The column temperature was programmed from 210 to 250 °C at a linear rate of 20 °C/min. The initial and the final hold-up times were 12 and 8 min, respectively. The injector and the detector were set at 290 °C and 300 °C, respectively. One µL methyl ester was injected into the column in split mode (split ratio 50:1). The fatty acid composition was reported as relative percentages of the total peak area, from 3 replicates.

2.5 Oil body isolation and analysis

2.5.1 Oil body extraction

Physic nut oil bodies were isolated and purified using two-layer flotation by centrifugation, detergent washing and ionic elution according to the published procedures (Tzen, Peng, Cheng, Chen, & Chiu, 1997).

2.5.2 Fluorescence microscopic observation

The purified oil bodies were stained with 7.85 µM Nile red solution, in dark, for 20 min and visualized using an Axioskop 2 Plus microscope (Zeiss, Germany) equipped with a charge-coupled device camera (Coolsnap-Prock, Photometrics Ltd., USA) (Lin, Jiang, Chen, & Tzen, 2012). The diameters of oil bodies were measured from 100 oil body samples using ImageJ program.

2.5.3 Neutral lipid analysis by TLC

Neutral lipids in the oil bodies were extracted and analyzed using TLC according to the procedures described by Lin *et al*. (2012). After extraction with chloroform/methanol, the chloroform layer was collected for TLC analysis. The samples and TAG marker were spotted onto TLC plate which was previously developed to the top (R_f = 1) in hexane: diethyl ether: acetic acid (70: 30: 1 v/v/v). The plate was air-dried at

room temperature and then developed in benzene to the $R_f = 1$ position. The plate was developed to $R_f = 0.5$ in hexane: diethyl ether: acetic acid (70: 30: 1 v/v/v) followed by staining with 0.03% Coomassie Brilliant Blue R-250.

2.5.4 Oil body protein analysis by SDS-PAGE and Western blotting

The SDS-PAGE was performed with minor modifications to a previous report (Lin *et al*., 2012). Oil body proteins were extracted with 4× sample buffer before resolved by SDS-PAGE utilizing 15% polyacrylamide separating gel and 4.75% polyacrylamide stacking gel. After electrophoresis, the gel was stained with 0.125% Coomassie Brilliant Blue R-250 and destained with a solution containing methanol and acetic acid. For Western blotting, proteins were transferred from SDS-PAGE onto nitrocellulose membranes in a Trans-Blot system (Bio-Rad, USA). The membranes were incubated in the primary antibodies against sesame seed caleosin (27 kDa), high molecular weight oleosin (oleosin-H, 17 kDa) or low molecular weight oleosin (oleosin-L, 15 kDa) and afterwards in the second antibodies conjugated with anti-rabbit for the caleosin or anti-chicken alkaline phosphatase for the oleosins (Pasaribu *et al*., 2014). The membrane color was developed in dark using the developmental buffer supplemented with substrates nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

2.5.5 Oil body protein analysis by LC-MS/MS

Physic nut oil body proteins resolved by SDS-PAGE were excised from the gel and destained in a solution containing 50% acetonitrile and 50 mM ammonium bicarbonate. They were reduced and alkylated with 10 mM dithioerythreitol and 55 mM iodoacetamide in 50 mM ammonium bicarbonate respectively before digesting with trypsin at 37 °C overnight. The tryptic peptides were extracted twice with 50% acetonitrile and 1% formic acid. The samples were analyzed by LC-MS/MS on UltiMate 3000 RSLCnano LC Systems (Thermo Fisher Scientific) using an Acclaim PepMap C18 column (75 µm I.D. x 25 cm nanoViper, Thermo Fisher Scientific). The mobile phase consisted of solvents A (ddH_2O containing 0.1% formic acid) and B (100% acetonitrile with 0.1% formic acid). Ten µL samples were loaded in the column, which was eluted at a flow-rate of 300 nL/min. The eluent was then introduced to TripleTOF® 6600 system mass spectrometer (Applied Biosystems Sciex) operating in electrospray ionization (ESI) mode. The MS/MS data were subjected to search against the Swiss-Prot protein sequence database using Mascot software (Matrix Science Ltd., UK).

2.6 Data analysis

Significant differences among treatments were considered using Kruskal-Wallis test. All significant differences were determined at a significance level of 0.05 using portable IBM SPSS statistics software version 19 (SPSS Inc., USA) software. Fruit diameter was fit with the following logistic function modified from Wubs *et al*. (2012) using Excel Solver (Microsoft Excel 2010).

$$Y = \frac{A}{1+ e^{-k(x-x_m)}} \ ;$$

where Y is the fruit diameter at time after pollination, A is the upper limit of fruit diameter, k is the constant defining the curvature of the growth feature, x is time after pollination, and x_m is the position of the inflection point where the growth rate is highest.

3. Results and Discussion

3.1 Pollination methods affecting fruit set, seed number and seed oil accumulation

The *Jatropha* flowers grown in the plantation were often visited by plenty of honey bees (Apidae Family). Among all pollination routes, open pollination exhibited the highest fruit set (74.33 ± 4.52%) and the maximum seeds per fruit (2.90 ± 0.08) (Table 1). The maximal fruit set and seed numbers were reported for open pollination in this *Jatropha* species grown in Bengaluru and India (Kaur *et al*., 2011; Pranesh *et al*., 2010).

Table 1. Fruit set, seed number and seed oil features of *Jatropha curcas* among four pollination methods

	Open pollination	Cross-pollination	Self-pollination	Non-pollination (Apomixis)
Fruit set (%)	74.33 ± 4.52 ^{a}	48.90 ± 4.12 ^{b}	31.10 ± 5.07 ^{c}	14.04 ± 2.73 ^{d}
Number of seeds per fruit	2.90 ± 0.08 ^{a}	2.29 ± 0.12 ^{b}	2.23 ± 0.12 ^{bc}	1.94 ± 0.12 ^{c}
Oil content in seed (%)	28.76 ± 1.60 ^{ns}	25.62 ± 1.05 ^{ns}	22.90 ± 0.13 ^{ns}	23.09 ± 0.22 ^{ns}
Fatty acid (% methyl ester)				
Palmitic acid (C16:0) *	13.04 ± 0.01 ^{d}	13.50 ± 0.00 ^{a}	13.33 ± 0.00 ^{c}	13.47 ± 0.01 ^{b}
Stearic acid (C18:0)	7.65 ± 0.00 ^{a}	7.13 ± 0.00 ^{b}	6.87 ± 0.00 ^{d}	7.10 ± 0.00 ^{c}
Oleic acid (C18:1)	48.55 ± 0.02 ^{a}	48.16 ± 0.02 ^{b}	47.82 ± 0.01 ^{c}	47.16 ± 0.02 ^{d}
Linoleic acid (C18:2)	26.57 ± 0.01 ^{d}	27.42 ± 0.01 ^{c}	28.33 ± 0.01 ^{b}	28.64 ± 0.01 ^{a}
Other fatty acids	1.61 ± 0.00 ^{d}	1.74 ± 0.02 ^{b}	1.81 ± 0.00 ^{a}	1.70 ± 0.00 ^{c}
SFA	21.22 ± 0.01 ^{a}	21.17 ± 0.03 ^{a}	20.77 ± 0.01 ^{c}	21.10 ± 0.01 ^{b}
MUFA	49.45 ± 0.02 ^{a}	49.11 ± 0.02 ^{b}	48.80 ± 0.02 ^{c}	48.09 ± 0.02 ^{d}
PUFA	26.76 ± 0.01 ^{d}	27.66 ± 0.01 ^{c}	28.60 ± 0.01 ^{b}	28.87 ± 0.01 ^{a}

Values are mean ± S.E.
Within a row, means followed by the same letter are not significantly different at a significance level of 0.05.
ns = no significant difference
* number of carbon chain length: number of unsaturated bonds.
MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid

These results may reflect the role of pollinators and pollen resources on increasing fruit and seed set in open pollination. Numerous bees found in our study field were likely to help this plant species improve the fruit quality by increasing pollen deposition and competition in the natural cross treatment (Roldán-Serrano & Guerra-Sanz, 2006). It has been established that the bees of Apidae (*Xylocopa confuse*, *Apis cerana* and *A. dorsata*) are effective pollinators for *J. curcas* as often visiting the plants and enhancing fruit and seed set (Rianti, Suryobroto, & Atmowidi, 2010).

No significant differences in seed oil content were found among the pollination treatments, although the open pollination seemed to have the maximal level (28.76 ± 1.60%) (Table 1). Recently, the highest oil yield was obtained in *J. curcas* grown in Zambia after open pollination (Negussie *et al*., 2015). Besides, regarding our study and that of Negussie *et al*. (2015), a relationship between oil accumulation and breeding mode has hardly been studied in *J. curcas* – most studies are restricted on oil related to natural-pollinated condition only. For example, an equivalent 31.6% seed oil content was reported for *J. curcas* grown in Brazil (Oliveira et al., 2009) and China (Pan & Xu, 2011) and around 29.85 - 37.05% were reported for those cultivated in India (Rao, Korwar, Shanker, & Ramakrishna, 2008).

The major fatty acids found in *Jatropha* oil were oleic acid, linoleic acid, palmitic acid and stearic acid (Table 1). Among the fatty acids, oleic acid was the most predominant, contributing nearly half of all fatty acids (47.16 ± 0.02 - 48.55 ± 0.02%). This study showed that the breeding mode affected the proportions of fatty acids but not to the type of fatty acids. The open pollination provided the best characteristics of oil with the greatest content of oleic acid (48.55 ± 0.02%) and thus the most monounsaturated fatty acid (MUFA, 49.45 ± 0.02%). This high content of oleic acid will give biodiesel with high quality in the critical parameters, such as cetane number, cold filter plugging point, and iodine value (Ramos, Fernandez, Casas, Rodriguez, & Perez, 2009). Therefore, *Jatropha* oil obtained from open pollinated seeds may be a good source for biodiesel production. Accordingly, the open pollination method, presenting the highest fruit set, seed number and oleic acid content, was employed in further analysis of the development of fruit diameter, embryo and endosperm, as well as of lipid storage in the embryo and endosperm.

3.2 Growth of fruit, embryo and endosperm

Fruit diameter was fit with a logistic model, with a high coefficient of determination (0.90) (Figure 1). The logistic model equation was $Y = \frac{29.97}{1+e^{-0.27(x-15.41)}}$. The simple sigmoid curve showed five phases, namely lag, exponential, linear, deceleration, and stationary phases at 0 - 3, 3 - 12, 12 - 21, 21 - 30, and 30 - 48 DAP, respectively. During the lag to exponential phase, the fruits (2.81 to 8.14 mm in diameter) consisted of proembryo (Figure 2A) and free nuclear endosperm (Figure 2B). In the linear phase, with the fruit sizes ranging between 8.14 and 24.66 mm, the embryo was globular in shape and the endosperm could be at both free-nuclear and cellular stages. At 18 DAP of the linear phase (20.18 mm fruit), as the embryo was still of globular shape (Figure 2C), cellularization of some endosperm cells located at micropylar region was noticeable (Figure 2D). The deceleration phase took place when the fruit reached 24.66 - 29.31 mm and possessed heart- or torpedo-shaped embryos. These heart (Figure 2E) and torpedo embryos (Figure 2G) could be observed at 21 - 24 DAP (24.66 - 28.26 mm) and 24 - 30 DAP (28.26 - 29.31 mm), respectively. Their complete cellular endosperm cells displayed undetectable storage globule (Figure 2F and H). Mature embryo and thick endosperm (Figure 2I) were noticeable at stationary phase with fruit diameters between 28.81 to 30.30 mm. Contrarily to those found at heart and torpedo embryo stages, the mature endosperm cells tangibly presented many storage globules (Figure 2J). As confirmed by Oil Red O staining, both embryo (Figure 2K) and endosperm (Figure 2L) certainly served as oil storage sites at maturity.

This study, for the first time in *J. curcas*, illustrated that the fruit diameter growth, a nondestructive assessment, was well fit with a logistic function (R^2 = 0.90), indicating the efficiency of the model for describing biological incidents related to fruit development (Merchán, Arévalo, Cely, Pinzón, & Serrano, 2016). The slow growth of *Jatropha* fruit size at the initial stage (0 - 9 DAP) could be characterized by high cellular division, while the rapid growth during 9 - 21 DAP corresponded to a cell elongation process (Hernández & Hernández, 2012). Since cell division and elongation were the crucial factors defining the final fruit size, which is strongly linked to final yield, sufficient care (i. e. irrigation and fertilizer application to support cell division and elongation) during 0 - 21 DAP might improve the physic nut productivity.

3.3 Oil content and fatty acid composition in embryo and endosperm

The oil content was higher in the endosperm (53.04 ± 1.43%) than in the embryo (48.37 ± 0.34%) (Table 2). This is in agreement with prior research by Sood and Chauhan (2015) who reported that TAG biosynthesis-related genes were more expressed in the endosperm than in the embryo, for the same species. Ten fatty acids were discovered in the isolated embryo and endosperm instead of the fourteen fatty acids found in the whole seed – lacking behenic, erucic, lignoceric and nervonic acids (just around 0.2% in total) in embryo and endosperm (Table 2). The presence of behenic, erucic, lignoceric and nervonic acid in the whole seed (with seed coat) but not in the isolated embryo and endosperm (without seed coat) indicates

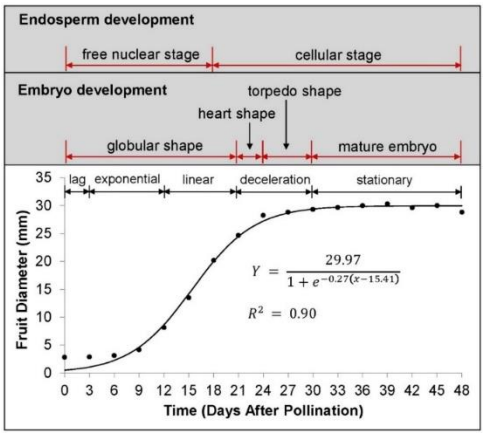

Figure 1. Sigmoidal pattern of fruit growth in relation to embryo and endosperm development obtained with open pollination.

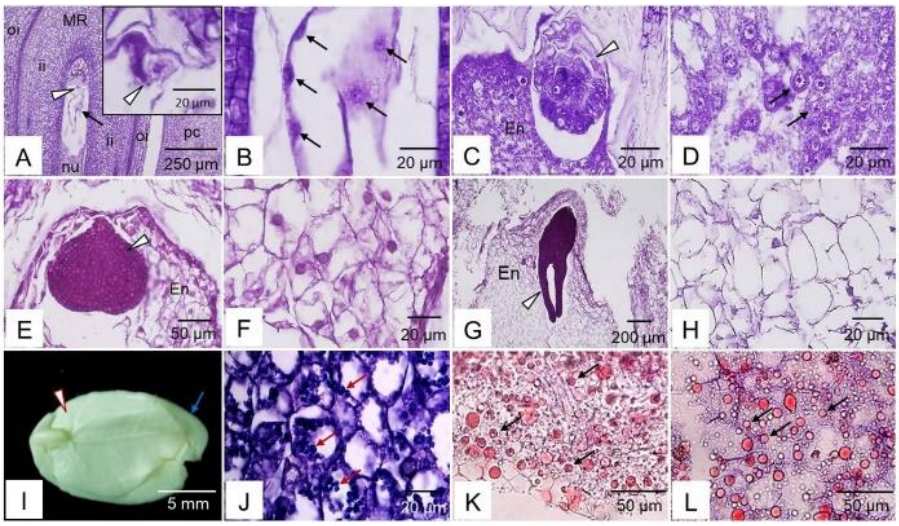

Figure 2. Embryo and endosperm development

Table 2. Characteristics of extracted oil derived from whole seed, embryo and endosperm

Characteristics	Whole seed*	Embryo	Endosperm
Oil body diameter (µm)	2.52 ± 0.08^{ns}	2.84 ± 0.12^{ns}	2.64 ± 0.11^{ns}
Oil content (%)	28.76 ± 1.60^{c}	48.37 ± 0.34^{b}	53.04 ± 1.43^{a}
Fatty acid (%)			
Myristic acid (C14:0)	0.05 ± 0.00^{c}	0.11 ± 0.00^{a}	0.06 ± 0.00^{b}
Pentadecanoic acid (C15:0)	0.02 ± 0.00^{b}	0.03 ± 0.00^{a}	0.02 ± 0.00^{b}
Palmitic acid (C16:0)	13.04 ± 0.01^{b}	10.35 ± 0.01^{c}	13.93 ± 0.01^{a}
Palmitoleic acid (C16:1)	0.80 ± 0.00^{b}	0.10 ± 0.00^{c}	0.87 ± 0.00^{a}
Heptadecanoic acid (C17:0)	0.11 ± 0.00^{b}	0.15 ± 0.01^{a}	0.11 ± 0.00^{b}
Stearic acid (C18:0)	7.65 ± 0.00^{b}	13.57 ± 0.01^{a}	6.78 ± 0.00^{c}
Oleic acid (C18:1)	48.55 ± 0.01^{a}	38.41 ± 0.03^{c}	45.95 ± 0.04^{b}
Linoleic acid (C18:2)	26.57 ± 0.01^{c}	36.19 ± 0.03^{a}	31.84 ± 0.04^{b}
Linolenic acid (C18:3)	0.19 ± 0.00^{b}	0.73 ± 0.02^{a}	0.16 ± 0.01^{c}
Arachidic acid (C20:0)	0.24 ± 0.00^{a}	0.22 ± 0.00^{b}	0.08 ± 0.00^{c}
Behenic acid (C22:0)	0.05 ± 0.00^{a}	0	0
Erucic acid (C22:1)	0.03 ± 0.00^{a}	0	0
Lignoceric acid (C24:0)	0.06 ± 0.00^{a}	0	0
Nervonic acid (C24:1)	0.06 ± 0.00^{a}	0	0
SFA	21.22 ± 0.01^{b}	24.44 ± 0.01^{a}	21.00 ± 0.01^{c}
MUFA	49.45 ± 0.02^{a}	38.51 ± 0.03^{c}	46.82 ± 0.04^{b}
PUFA	26.76 ± 0.01^{c}	36.92 ± 0.05^{a}	32.00 ± 0.03^{b}

Values are mean ± S.E.
Within a row, means followed by the same letter are not significantly different at a significance level of 0.05.
ns, no significant difference
* With seed coat
MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid

that those minor fatty acids possibly located in the seed coat, especially in the shrunken layer-like tissue developed from inner integument. To support this idea, Chaitanya *et al.* (2015) showed that the inner integument, being a part of seed coat underlying the hardened seed coat derived from outer integument, of *J. curcas* could store 0.23 - 1.01% lipid throughout seed development.

Physic nut endosperm stored more oleic and palmitic acid but less stearic and linoleic acid than the embryo (Table 2). This result is different from that for *Argania spinosa* in which the endosperm was higher in linoleic but lower in oleic, palmitic and stearic acids than the embryo (Errouane *et al*., 2015). In *Elaeis guineensis*, nonetheless, the embryo contained more oleic, palmitic, linoleic and stearic acid than the endosperm (Dussert *et al*., 2013). These differences in lipid allocation between embryo and endosperm, in diverse plants, might correlate to tissue-specific transcriptional specialization (Dussert *et al*., 2013). Noticeably, the high proportion of oleic acid in comparison to other fatty acid types in both embryo and endosperm represents the potential for qualified biodiesel production, since that kind of fatty acid provides overall good properties to biodiesel, including cetane number, iodine value and cool filter plugging point (Ramos *et al*., 2009).

3.4 Characteristics of oil bodies isolated from the whole seed, embryo and endosperm

Oil bodies isolated from all seed tissues formed a milky layer during extraction (Figure 3A). The oil body diameters of the whole seed (2.52 ± 0.08 µm), embryo (2.84 ± 0.12 µm) and endosperm (2.64 ± 0.11 µm) were not significantly different (Table 2) and close to the range of 0.5 - 2.5 µm generally found in other species (Tzen *et al*., 1993).

Neutral lipids, presented in oil bodies that were isolated from the seed tissues, were detected from their red-color inflorescence after Nile red staining (Figure 3B-G) and they were chiefly TAG as confirmed by TLC analysis (Figure 3H). Four bands of oil body protein (27, 17, 15 and 14 kDa) were mainly found in the seed of *J. curcas* (Figure 4A). The 27-kDa protein found in physic nut oil bodies could be cross-recognized by antibodies against sesame caleosin (Figure 4B). However, no immunological cross-recognition was detected by antibodies against sesame oleosin-H (Figure 4C) and oleosin-L (Figure 4D) under our experimental conditions. This non-cross recognition of *J. curcas* oleosins by the antibodies against sesame oleosins might be because the sequence homology of *J. curcas* and sesame oleosins is located in the central hydrophobic domain but not in the hydrophilic region including N- and C-terminal domains (Tzen, Chuang, Chen, & Wu, 1998).

The LC-MS/MS results showed that the protein of 27, 17, 15 and 14 kDa highly matched the hypothetical proteins JCGZ_02382 (caleosin, accession no. KDP40384), oleosin 2 (H-form, accession no. ABW90149), oleosin 3 (L-form, accession no. ABW90150) and 14.3 kDa oleosin (L-form, accession no. AFP19885) of *J. curcas* in the database, by producing 8, 5, 3 and 2 matched peptide residuals, respectively (Table 3). Therefore, two isoforms of oleosins, the H- and the L-form, were examined in this study and these proteins, especially oleosin 3 (L-form), were the major proteins found in both embryo and endosperm of physic nut. This was consistent with the earlier finding that oleosins were found in both embryo and endosperm of this species after two-dimensional gel electrophoresis (Liu, Liu, Yang, & Shen, 2009) and transcriptional expression of oleosin 3 was higher than that of other oleosins (Popluechai *et al*., 2011). The oleosin 3 might have potential for use as a codominant marker in breeding for improving seed oil characteristics in this species (Popluechai *et al*., 2011). Oleosin proteins play an important role in preventing coalescence of lipid bodies during seed dehydration, imbibition and germination, and they control the oil body size (Siloto *et al*., 2006). The suppression of these proteins changed the oil body sizes, which resulted in the reduction of lipid content and modified the fatty acid components in *Arabidopsis* seeds (Siloto *et al*., 2006).

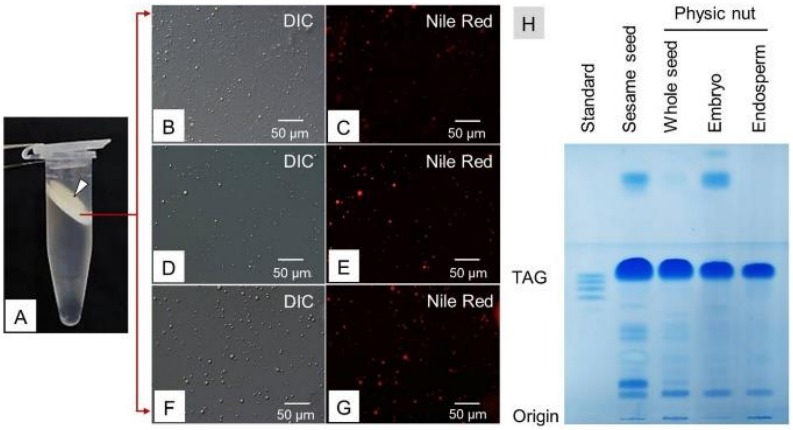

Figure 3. Physic nut seed oil body and neutral lipid analysis

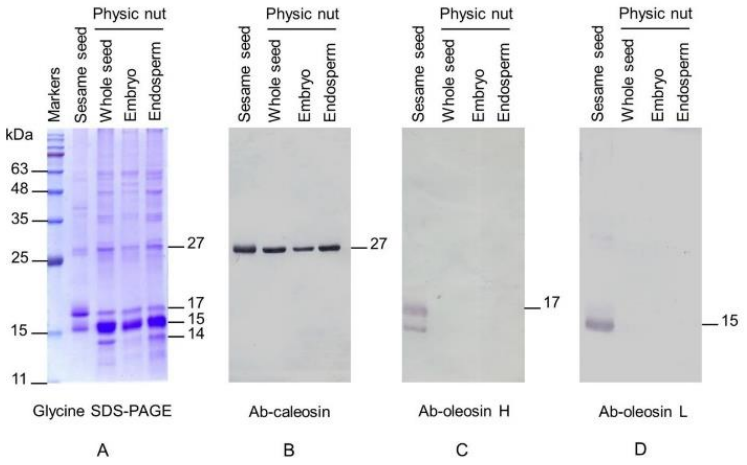

Figure 4. SDS-PAGE and Western blots of oil body protein extracted from physic nut seed.

Table 3. Fragments of proteins found in purified oil bodies of physic nut identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Apparent mass (kDa)	Identified protein	Residuals	Sequences
27	Hypothetical protein JCGZ_02382 (caleosin)	5-11	TDDSLDR
		12-39	AAPFAPVTFHRPVRDDLET TLPKPYMAR
		40-56	ALQAPDTEHPQGTPGHK
		132-146	AKHGSDSGTYDTEGR
		147-158	YMPVNLENIFSK
		204-211	DEEGFLSK
		216-228	RCFDGSLFEYCAK
		229-238	MNMGSESKMY
17	Oleosin 2 (H-form)	18-23	YEAAFK
		110-116	YLQEVTR
		118-126	MPEQLDIAK
		129-139	MQDMAGFVGQK
		140-149	TKEVGQEIQR
15	Oleosin 3 (L-form)	1-15	MAEHPQSQHVGQQPR
		100-111	HPPGAENLDQAR
		123-137	DRAEQFGQHVTGQQT
14	14.3 kDa oleosin (L-form)	1-15	MAEHPQSQHVGQQPR
		125-137	AEQFGQHVTGQQT

H-form, high molecular weight isoform
L-form, low molecular weight isoform

4. Conclusions

The open pollination proved to be the best pollination method for physic nut as it provided maximal fruit set, number of seeds per fruit and the most monounsaturated oleic acid. This study also gives novel information on the growth of fruit diameter, embryo and endosperm in association to the time after pollination, well fit with a logistic function. Moreover, the endosperm was the main source of oil and oleic acid content in the seed. Oil bodies from both embryo and endosperm showed similar characteristics, comprising TAG enclosed by integral proteins termed caleosin, high molecular weight oleosin and low molecular weight oleosin.

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