Establishment of Method for Protoplast Fusion with PEG-mediated between *Jatropha curcas* L. and *Ricinus communis* L.

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***Abstract*—*Jatropha curcas* L. and *Ricinus communis* L. belong to Euphorbiaceae and considered as important alternative biofuel. Somatic hybridization between *J. curcas* L. and *R. communis* L. was proposed to solve low genetic variability of *J. curcas* L. The intergeneric hybrid will provide a novel variety which perform as an annual crop and bear fruits to ease harvesting for commercial production. Protoplast fusion between *J. curcas* L. and *R. communis* L. to create intergeneric hybrid was attempted via PEG-mediated method. Mesophyll protoplasts of *J. curcas* L. and calli protoplasts of *R. communis* L. were used in this study. Concentration and molecular weight of PEG, fusion period, microfusion and macrofusion method were optimized. The highest (66%) viability of heterokaryon was obtained from using 30% PEG (MW6000) assisted with macrofusion method for 10 minutes.**

***Index Terms*—*Jatropha curcas* L., *Ricinus communis* L. protoplast fusion, PEG-mediated method.**

I. Introduction

Physic nut (*Jatropha curcas* L.) and castor bean (*Ricinus communis* L.) are important non-edible oilseed crops in the Euphorbiaceae. *J. curcas* L. seed is alternative source of biofuel; while *R. communis* L. is an important source of medicinal oil and has numerous benefits to humanity [1]. Thus, both species are considered potential alternative sources for biofuel production augment world energy crisis, providing opportunities for current breeding programs in genetic enhancement of the oilseed crops [1], [2]. Intergeneric somatic hybridization between *J. curcas* L. and *R. communis* L., using protoplast fusion can be an alternative way to introgress desirable characters of related family in obtaining superior genotype and converting an underutilized wild type to a commercially viable alternative source of biofuel, thereby increasing new valuable annual crops in support for the growing plant breeding program in Thailand. The polyethylene glycol (PEG)-mediated method is one of the most successful techniques for protoplast fusion that attempts to enhance agglutination to produce intergeneric somatic hybrids. PEG-mediated method has been used extensively for its simplicity, efficiency, economical, and does not seem to interfere with protoplast viability [3], [4]. However, protoplast fusion requires the establishment of an efficient system of protoplast isolation followed by cell division and plant regeneration [5]. There are no reports to date describing the fusion of protoplast of *J. curcas* L. and *R. communis* L. Therefore the development of techniques for protoplast fusion between *J. curcas* L. and *R. communis* L. by PEG-mediated was investigated to find the optimum condition for protoplast fusion of the intergeneric somatic hybridization of the two species.

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II. Materials and Methods

A. Plant Materials

In vitro leaves of *J. curcas* L. were precultured for 2 days on modified MS medium [6] supplemented with 100 mg/l casein hydrolysate, 200 mg/l L-glutamine as an organic nitrogen source, 0.5 mg/l IBA, 1.0 mg/l BA, and 8.0 mg/l $CuSO_4$; while *in vitro* leaves of *R. communis* L. were precultured on modified MS medium supplemented with 0.25 mg/l *N*-phenyl-*N'*-1,2,3-thidiazol-5-yl urea (TDZ), 4.5 mg/l BA, 3% (w/v) sucrose, and 0.7% (w/v) agar, adjusted the pH to 5.7. Compact calli of *R. communis* L. were derived from *in vitro* leaves growing on modified MS medium supplemented with 0.6 mg/l of TDZ, 0.05 mg/l of napthalene acetic acid (NAA), 1 mg/l of BA, 3% (w/v) sucrose, and 0.7% (w/v) agar, adjusted to pH 5.7, and cultured for 6 weeks.

B. Protoplast Isolation and Purification

Protoplasts were isolated from *J. curcas* L. and *R. communis* L. as described [7]. Protoplasts were freshly prepared prior to fusion experiment.

C. Protoplast Fusion

The different fusion conditions were evaluated on the basis of four parameters: the PEG molecular weight, compositions of fusion solution, fusion time, and fusion methods. The fusion process was observed microscopically to estimate the percentage of binary and multi fusions, heterokaryons and cell viability after 12-24 h of fusion.

Effect of fusion solution and fusion time—Equal volumes of purified mesophyll protoplasts (green) of *J. curcas* L. and calli protoplasts (colorless) of *R. communis* L. were mixed in the ratio of 1:1 (v/v) density were adjusted to $1x10^5$ protoplasts/ml, and resuspended in CPW13M solution [8]. 100 µl per drop of protoplast suspension was transferred into each microcentrifuge tube, centrifuged at 750 rpm, 10 ◦C for 25 min., and slowly removed 90 µl of CPW13M solution with a Pasteur pipette. 100 µl of fusion solution (Table I) was gently added immediately to each microcentrifuge tube and incubated for 15, 20 and 25 min. Then the fusion solution was carefully and slowly removed with a Pasteur pipette and immediately replaced with 400 µl of High pH/Ca^{2+} (high pH/high Ca^{2+}) solution (Table I) and mixed gently (1:1, v/v) before adding to each microcentrifuge tube. After 10 min, fusion solution and high pH/Ca^{2+} solution were centrifuged at 750 rpm, 10 ◦C for 5 min and slowly removed with pasture pipette, and immediately replaced with 1 ml of CPW13M (pH 5.8). This step was repeated more than 3 times, with great care to avoid removing protoplasts.

TABLE I. COMPOSITION OF FUSION SOLUTION AND HIGH PH/CA^{2+} SOLUTION (PH 10.5)

Composition	Fusion solution					High pH/Ca^{2+} (pH 10.5)
	1	2	3	4	5	
PEG MW 6000 (% w/v)		15	30	40	50	
Sucrose (% w/v)	5	60	4 10	10.5	0.5	100
$CaCl_2.2H_2O$ (mM)				0.7		
$KH_2PO_4.H_2O$ (mM)	500	90			500	800
Mannitol (mM)	3.753	25				100
Glycine (mM)						
Reference	[9]	[10]	[9]	[11]	[12]	

Effect of molecular weight of PEG—To compare different molecular weight (MW) of 30% (w/v) PEG between MW6000 and MW8000 along with 4% (w/v) sucrose and 10 mM $CaCl_2.2H_2O$ modified from Ref. [9], equal volumes of purified mesophyll protoplasts of *J. curcas* L. and calli protoplasts of *R. communis* L., at density adjusted to $1x10^5$ protoplasts/ ml were mixed in the ratio of 1:1 (v/v). Protoplast suspension (100 µl) was transferred into each new microcentrifuge tubes and mixed with the fusion solution (MW6000 and MW8000) and centrifuged at 750 rpm, 10 ◦C for 25 min. 400 µl of high pH/Ca^{2+} solution was added to each microcentrifuge tubes. After 10 min, fusion solution and high pH/Ca^{2+} solution were centrifuged at 750 rpm, 10 ◦C for 5 min, the method was performed as described in former experiment.

Effect of fusion methods—(1) Macromethod: After isolation, Equal volumes of purified mesophyll protoplasts (green) of *J. curcas* L. and calli protoplasts (colorless) of *R. communis* L. at density $1x10^5$ protoplasts/ml were mixed in the ratio of 1:1 (v/v). Protoplast suspension (100 µl) was transferred into new microcentrifuge tubes and mixed with the fusion solution 3 containing 30% (w/v) PEG-MW6000 along with 4% (w/v) sucrose and 10 mM $CaCl_2.2H_2O$ and induced fusion by centrifugation at 750 rpm, 10 ◦C for different times (10, 15, 20, 25 and 30 min). After centrifugation, the mixture was carefully diluted with 400 µl (1:1, v/v) of high pH/Ca^{2+}. After 10 min, fusion solution and high pH/Ca^{2+} solution were centrifuged at 750 rpm, 10 ◦C for 5 min the method was performed as described in former experiment. *(2) Micromethod*: After isolation, Equal volumes of the two fusion partners (1:1, v/v), at density $1x10^5$ protoplasts/ml were mixed in CPW13M. Protoplast suspension (100 µl) was pipetted in small droplets transferred into each 5.5 cm Pretri dish. After settling of protoplasts for at least 20 min (to allow accumulation of protoplasts in the center of the drops), 100 µl of fusion solution 3 containing 30% (w/v) PEG-MW6000 along with 4% (w/v) sucrose and 10 mM $CaCl_2.2H_2O$ was slowly added on top of the protoplast mixture and allowed to stand at room temperature for different time (10, 15, 20, 25 and 30 min). After incubation time, the mixture was carefully diluted with 400 µl of high pH/Ca^{2+} solution. After 10 min, the mixture of fusion solution and high pH/Ca^{2+} solution were carefully and slowly removed with Pasteur pipette, and immediately replaced with 1 ml of CPW13M (pH 5.8). The method was performed as described in former experiment. About 12-24 h after culture in CPW13M (pH 5.8) estimated percentage of binary and multi fusions, heterokaryons formation and cell viability were determined by observing under the compound microscope (Olympus CX31, USA).

D. Statistical Analysis

All data were assessed by one way analysis of variance (ANOVA), and the means were compared by the Duncan's multiple range test at 95% interval of confidence ($P<0.05$). Each statistical analysis was carried out using SPSS 17.0 software (SPSS, Chicago. IT. USA).

III. RESULTS

A. Protoplast Preparation

Protoplasts of *J. curcas* L. were released from *in vitro* leaves after incubation for 7 h with combination of 2% (w/v) cellulase onozuka R10, and 0.2% (w/v) pectolyase Y23 dissolved in 0.7 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM MES buffer (pH 5.6), while protoplasts of *R. communis* L. were released from callus after incubation for 7 h with combination of 2% (w/v) cellulase onozuka R10, and 2% (w/v) pectinase dissolved in 0.5 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM MES buffer (pH 5.6). After purification, the preparation frequency of protoplasts of *J. curcas* L. and *R. communis* L. came to the maximum 5.45×10^6 and 1.77×10^5 protoplasts/gFW, respectively. The freshly protoplasts of *J. curcas* L. were green, spherically shaped, and heterogeneous in size (range: 11-45 µm, Fig. 1C). On the other hand, the callus protoplasts of *R. communis* L. were translucent, colorless, and vary in sizes (range: 36-97 µm, Fig. 1D). This experiment demonstrated that the isolated protoplasts of *J. curcas* L. and *R. communis* L. gave viability 77.03 and 82.54 % of protoplasts, respectively.

B. Effect of Different Fusion Solution and Fusion Times on Protoplast Fusion

Different combinations of five fusion solutions behaved differently in their responses to varying PEG, sucrose, $CaCl_2.2H_2O$, $KH_2PO_4.H_2O$, mannitol and glycine concentration. After the treatment, most of the cells exhibited binary fusion (Fig. 1E, G), while multi fusion was rarely observed in some case as three-five protoplasts were also merged (Fig. 1F). Homokaryons products could be not identified due to their monochromatic nature (Fig. 1E). While, heterokaryons products could be easily identified by counting the protoplasts present in both green chloroplasts of mesophyll-derived protoplasts partner from *J. curcas* L. and colorless chloroplasts of calli-derived protoplasts partner from *R. communis* L. (Fig. 1G). In our study, the highest binary fusion rate (6.42±1.44%) was achieved with Fusion solution 3 containing 30% (w/v) PEG-MW6000 supplement with 4% (w/v) sucrose and 10 mM $CaCl_2.2H_2O$ and incubated in high pH/Ca^{2+} solution (pH 10.5). Viability of protoplast mixture was measured after 24 h of fusion (Table II). The highest viability rate was obtained (80.07±2.97%) when protoplasts were treated with Fusion solution 4 containing 40% (w/v) PEG-MW6000 supplement with 10.5 mM $CaCl_2.2H_2O$ and 0.7 mM $KH_2PO_4.H_2O$, centrifuged at 750 rpm, 10 °C for 25 min . In this study, Fusion solution 3 containing 30% (w/v) PEG-MW6000 supplemented with 4% (w/v) sucrose and 10 mM $CaCl_2.2H_2O$ and incubated in high pH/Ca^{2+} solution (pH 10.5) can be considered the optimum combination to enhance protoplast fusion production between *J. curcas* L. and *R. communis* L.

C. Effect of Molecular Weight of PEG on Protoplast Fusion

In this study, protoplast fusion between *J. curcas* L. and *R. communis* L. used different molecular weight between PEG -MW 6000 and MW 8000 at concentration of 30% (w/v) supplemented with 4% (w/v) sucrose and 10 mM $CaCl_2.2H_2O$ and high pH/Ca^{2+} solution (pH 10.5) incubated and centrifuged at 750 rpm, 10 °C for 25 min modified from Ref. [9]. The effects of PEG-MW6000 and PEG-MW8000 on protoplast agglutination (tight adhesions between two or more protoplasts) was observed using hemocytometer. PEG-MW6000 was highly efficient in terms fused protoplast with about 6.42±1.44% of binary fusion (Table III) than PEG-MW8000, which only produced 3.13±0.26% successful fusion. In addition, PEG-MW6000 seemed to be more viable than PEG-MW8000 with viability rate of about 56.39±2.01% and 55.95±2.29%, respectively. Therefore, PEG-MW6000 was selected on protoplast fusion between *J. curcas* L. and *R. communis* L.

D. Effect of Protoplast Fusion Method and Time Duration

The efficiency of protoplast fusion between *J. curcas* L. and *R. communis* L. using PEG- mediated methods (macromethod and micromethod) were investigated. As the protoplast membrane fused and connections were formed between the two cytoplasms (Fig. 1E,G), it was observed that fusion methods has a significant effect on the frequency of binary fusion, multi fusion, heterokaryons and their viability (Table III). The percentage of binary fusion of macromethod was higher than micromethod (P<0.05) (Table III). However, micromethod resulted more viable fusion products than macromethod (P<0.05) due to the absence of centrifugation, which consistently caused damage to the membranes of the protoplasts [9]. Therefore, the longer time to produce protoplast fusion by macromethod led to serious protoplast damage more than short time. This study revealed that PEG-mediated by macromethod using 30% PEG-MW6000 supplemented with 4% (w/v) sucrose and 10 mM $CaCl_2.2H_2O$ and high pH/Ca^{2+} solution (pH 10.5) and centrifuged at 750 rpm, 10 °C for 10 min was the most efficient for fusing the protoplasts of *J. curcas* L. and *R. communis* L. Our investigation demonstrated that protoplast fusion between *J. curcas* L. and *R. communis* L. using macromethod at short time enhanced the frequency rate of binary fusion (7.62±5.19%), multi fusion (0.81±1.15%), heterokaryons (2.42±3.42%) and has high viability (66.89±17.04%). On the other hand, using micromethod at prolonged period produced high frequency of binary fusion but has lower viability.

IV. Discussion

Two protoplast fusion procedures have been used to obtain somatic hybrids produced to date-chemical and electrical fusion [13]. The protoplast using polyethylene glycol (PEG) requires the careful control of several important parameters, including the fusion media as well as the molecular weight and concentration of PEG as well as the duration of exposure and temperature [14], [15]. PEG having a molecular weight from 400 to 6000 was found to be active in fusion, whereas PEG 200 and 20000 was almost inactive. However, actual fusion occurs upon dilution of the PEG with a high pH and high Ca^{2+} eluting

medium, which has been shown to neutralize the normal surface charge thus allowing the membranes of agglutinated protoplasts to come in intimate contact [16].

TABLE II. EFFECTS OF DIFFERENT FUSION SOLUTION AND FUSION TIMES ON PROTOPLAST FUSION BETWEEN *J. CURCAS* L. AND *R. COMMUNIS* L.

Fusion solution	Fusion time (min)	Binary fusion rate (%)	Multi fusion rate (%)	Viability (%)
1	15	0.63 ± 0.11^{f}	0.00 ± 0.00^{b}	61.66 ± 2.67^{c}
	20	2.12 ± 0.56^{cdef}	0.00 ± 0.00^{b}	60.93 ± 1.54^{c}
	25	1.57 ± 1.85^{ef}	0.40 ± 0.69^{ab}	26.46 ± 1.40^{g}
2	15	0.76 ± 0.35^{f}	0.00 ± 0.00^{b}	74.48 ± 1.84^{b}
	20	0.82 ± 0.03^{f}	0.00 ± 0.00^{b}	49.03 ± 2.43^{e}
	25	3.63 ± 0.17^{bc}	0.60 ± 0.58^{b}	62.72 ± 1.66^{c}
3	15	0.68 ± 0.59^{f}	0.00 ± 0.00^{b}	62.30 ± 2.17^{c}
	20	1.84 ± 0.28^{def}	0.00 ± 0.00^{b}	61.92 ± 2.00^{c}
	25	6.42 ± 1.44^{a}	0.00 ± 0.00^{b}	56.38 ± 2.01^{d}
4	15	1.19 ± 0.03^{ef}	0.00 ± 0.00^{b}	80.07 ± 2.97^{a}
	20	2.88 ± 0.75^{bcde}	0.19 ± 0.33^{ab}	60.81 ± 2.61^{c}
	25	4.43 ± 1.75^{b}	0.00 ± 0.00^{b}	3.98 ± 0.43^{h}
5	15	0.99 ± 0.45^{f}	0.00 ± 0.00^{b}	36.95 ± 1.48^{f}
	20	3.44 ± 0.67^{bcd}	0.34 ± 0.58^{ab}	39.91 ± 3.38^{f}
	25	2.76 ± 1.35^{cde}	0.00 ± 0.00^{b}	37.41 ± 2.30^{f}

Data are means of three independent treatments. Differences between means were assessed using the Duncan's multiple range test; means with the same superscript letter are not significantly different at 95% interval of confidence ($P<0.05$).

TABLE III. EFFECT OF DIFFERENT MOLECULAR WEIGHT OF PEG ON PROTOPLAST FUSION BETWEEN *J. CURCAS* L. AND *R. COMMUNIS* L.

PEG MW	Protoplast fusion frequency (%)		Viability (%)
	Binary fusion	Multi fusion	
6000	6.42 ± 1.44^{a}	0.00 ± 0.00	56.39 ± 2.01^{a}
8000	3.13 ± 0.26^{b}	0.00 ± 0.00	55.95 ± 2.29^{a}

Data are means of three independent treatments. Differences between means were assessed using the Duncan's multiple range test; means with the same superscript letter are not significantly different at 95% interval of confidence ($P<0.05$).

TABLE IV. EFFECT OF FUSION METHODS AND FUSION TIME ON PROTOPLAST FUSION FREQUENCY (%) AND VIABILITY (%)

Fusion method	Fusion time (min)	Protoplast fusion frequency (%)			Viability (%)
		Binary fusion	Multifusion	Heterokaryons	
Macromethod	10	7.62 ± 5.19^{a}	0.81 ± 1.15^{a}	2.42 ± 3.42^{a}	66.89 ± 17.04^{abcd}
	15	4.56 ± 0.07^{ab}	0.51 ± 0.74^{a}	1.19 ± 0.25^{a}	71.85 ± 1.61^{abc}
	20	3.91 ± 2.56^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	66.66 ± 7.39^{abcd}
	25	3.32 ± 1.81^{ab}	0.00 ± 0.00^{a}	0.58 ± 0.81^{a}	64.40 ± 1.59^{bcd}
	30	1.55 ± 0.69^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	60.40 ± 3.53^{cd}
Micromethod	10	1.48 ± 0.13^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	77.87 ± 1.84^{ab}
	15	5.73 ± 2.31^{ab}	0.00 ± 0.00^{a}	1.25 ± 0.32^{a}	81.83 ± 5.50^{a}
	20	5.82 ± 0.90^{ab}	0.00 ± 0.00^{a}	0.97 ± 0.15^{a}	80.96 ± 5.00^{a}
	25	3.34 ± 0.49^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	66.67 ± 0.52^{abcd}
	30	6.86 ± 1.37^{a}	0.00 ± 0.00^{a}	1.36 ± 0.54^{a}	54.79 ± 1.22^{d}

Data are means of three independent treatments. Differences between means were assessed using the Duncan's multiple range test; means with the same superscript letter are not significantly different at 95% interval of confidence ($P<0.05$).

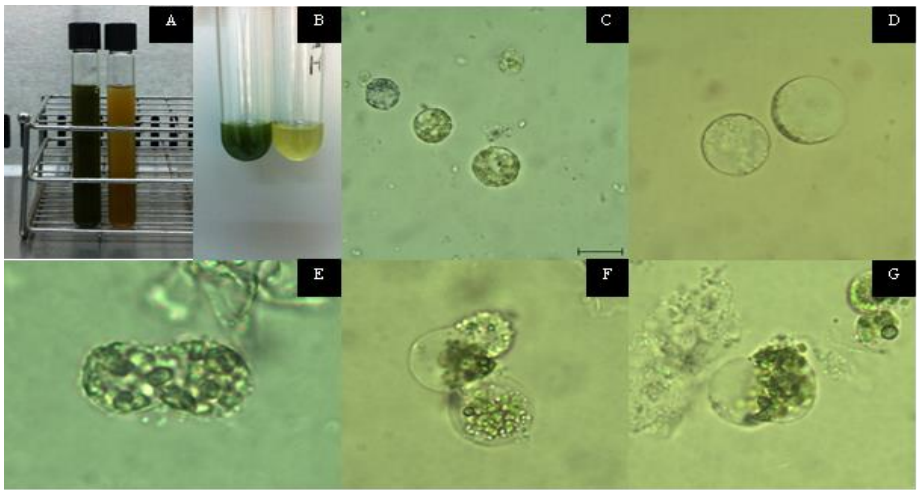

Figure 1. Protoplast fusion between *J. curcas* L. and *R. communis* L. protoplasts induced by 30% (w/v) PEG-MW 6000 supplemented with 4% (w/v) sucrose and 10 mM $CaCl_2.2H_2O$, isolated protoplasts of *in vitro* leaves of *J. curcas* L. and isolated protoplast of callus of *R. communis* L. (A), purified protoplast of *in vitro* leaves of *J. curcas* L. and purified protoplast of callus of *R. communis* L. (B), healthy protoplast isolated from *in vitro*

leaves of *J. curcas* L. (C), healthy protoplast isolated from callus of *R. communis* L. (D), Binary fusion (E), Multifusion (F) and heterokaryons between *J. curcas* L. and *R. communis* L. (G).

Each step in the fusion process should be evaluated not only for yield of fusion products, but also for its overall effect on cell viability [14]. In this study, viability of fused protoplast reached 26.46-80.07 %. The prolong PEG application of more than 30 min led to serious protoplast damage [17]. The use of higher concentration of PEG or higher molecular weight PEG is known to cause tighter adhesion of the protoplast. However, it also limits the recovery of viable fused cells. In this study, 50% Fusion solution promotes fusion, but it caused decreased viable fusion products. Moreover, is also important to identify the suitable molecular weight of PEG and exposure time can influence the yield of viable protoplast [15].

Ref. [15] reported that using PEG in prolonged period reduced the viability of protoplasts and cell wall regeneration, cell division and production of microcolonies. These results conformed to the previously published work of using 30% (w/v) PEG incubated to 30 min to induce fused protoplast to die [17]. One popular modification of chemical fusion protocols is the use of low-speed centrifugation during the aggregation and fusion stages. The increased centrifugal forces created by the centrifugation seem to promote tighter adhesion and promote higher yields of cells [14]. Similarly, we found that binary fusion frequency of macromethod was highly efficient than micromethod. In some case, a novel method known as electro-chemical protoplast fusion was developed which uses a low concentration of PEG induced protoplast aggregation and CD pulse to promote membrane fusion [18]. Meanwhile, agglutination of protoplasts treated with PEG could be highly dependent on the temperature of media in which the cells are suspended in, as well as the concentration and duration of the PEG treatment [14]. Following treatment with PEG, the mesophyll-derived protoplast continued to divide, resulting in cell colonies, whereas the calli-derived protoplasts died shortly after treatment. Genotype variation played a significant role and the use of a large number of different materials might be a needed to achieve not only regeneration from fused cells but also for chromosome elimination in somatic hybridization between remote species such as *Arabidopsis thaliana* L. and *Bupleurum scorzonerifolium* Willd. [19]. In essence, the observed slow division or slow growth of hybrids cells is attributed to loss of chromosomes, chromosome fragmentations or abnormal chromosome numbers [20].

In some cases, isolated protoplasts had completely lost the ability to regenerate the cell walls, divide and proliferate [21]. Ref. [22] reported that in the event of over-digestion by enzymes, the cultured protoplasts will not divide and cytoplasm may collapse within the cells. Therefore, developing an isolation procedure yielding to more viable and intact protoplasts may be necessary for protoplast division in protoplast culture step. Thus, the possible stimulatory potential Ca^{2+} ions to promote fiber formation of cell wall component [23], [24] and increased calcium concentration may be important for membrane stability [25]. However, high Ca^{2+} ions concentration at 900 mg/L $CaCl_2$ in protoplast culture medium (normal concentrations being 400 mg/l in MS medium) was effective for increasing protoplast division of *Nicotiana alata* [26] and the plating efficiency of Azuki bean leaf mesophyll protoplast [27]. Though the highest number of the Initial Plating Efficiency (IPE) was obtained in protoplast culture medium (PCM-6) but protoplasts did not divide and turned brown after 21 days of culture. Isolated protoplasts are usually cultured in either liquid or semisolid agar media plates. Ref. [25] reported that protoplasts are sometimes allowed to regenerated cell wall in liquid culture before they are transferred to agar media. The frequency of cell division and colony formation from protoplasts were each species despite the optimization of various nutritional factors [28].

Increasing $CaCl_2.2H_2O$ and mannitol concentration to prevent osmotic pressure and decreasing of PEG concentration can increase viability of fused protoplast. However, despite the high efficiency of increase PEG-MW6000 concentration for inducing binary fusion frequency it can result to low viable fusion products and can lead to perturbations of mitotic activities [12]. Our investigation demonstrated that Fusion solution without PEG (Fusion solution 1) has increased the binary fusion frequency but low viability (Table IV). The PEG molecule chain act as a molecule bridge between the surface of adjacent protoplasts and causes to adhere to one another while high pH/Ca^{2+} ion treatment has been shown to neutralize the normal surface charge causing agglutinated protoplasts to come in intimate contact [25], [16]. In addition, performing protoplast fusion by PEG-induced method within 1-2 h after protoplast isolation will allow cell wall regeneration and may hinder fusion [4], [29]. Protoplasts treated with PEG produced higher frequency of binary fusion than those fused electrically but has a lower rate of cell divisions [12]. Ref. [25] reported that the breakdown of cell wall during protoplast isolation and cell wall degradation would permit dilation of plasmodesmata leading to induce spontaneous fusion. Supporting the concept that sucrose plays an important role in membrane protection [30]. Ca^{2+} ion can become a fusagen by binding together the hydrogen phosphoric group on two different protoplasts though mutual attraction between the negative hydrogen phosphate group and the positive Ca^{2+} ion [31]. However, increased $CaCl_2$ concentration shown beyond a certain level the number of fusing protoplast.

In conclusion, this study is the first report on protoplast fusion of *J. curcas* L. and *R. communis* L. Although microcolony formation and plant regeneration was not achieved, our results provided a realistic basis for future work on the development of a protoplast to plant regeneration system. Protoplast culture of *J. curcas* L. and *R. communis* L. was cultured in liquid medium for 3-7 days for cell division followed by transferring to agarose droplet in the same medium for 42 days to induce microcolony. The optimum fusion solution that would

offer best compromise between high percentage of binary fusion and heterokaryons would be PEG-mediated by macrofusion method with 30% PEG-MW6000 supplement with 4% (w/v) sucrose and 10 mM $CaCl_2.2H_2O$, and incubated in high pH/Ca^{2+} solution (pH 10.5) and centrifuged at 750 rpm, 10 ◦C for 10 min. Further studies is recommended to improve plant regeneration by developing suitable protoplast culture and regeneration medium for somatic hybrids between *J. curcas* L. and *R. communis* L.

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