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Full Length Research Paper

Plant regeneration studies of *Jatropha curcas* using induced embryogenic callus from cotyledon explants

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A plant regeneration system for *Jatropha curcas*, a biofuel- producing plant, was established from the induced embryogenic callus. Cotyledon explants cultured on single auxin media were able to form callus. However, only the callus induced from cotyledon explants using Murashige and Skoog (MS) medium containing 0.8 mg l^{-1} dicamba was embryogenic. The somatic embryos were suitable to be proliferated using phytohormone- free woody plant medium (WPM). Two somatic embryo regeneration methods were studied and the direct somatic embryos plant regeneration method using the media containing 0.3 mg l^{-1} 6-benzylaminopurine (BAP) and 0.4 mg l^{-1} gibberellic acid (GA_3) was more effective, 83.3 and 73.33% plant regeneration, respectively. The other method that needed to mature the somatic embryos prior to plant regeneration from the phytohormone- free MS medium was not preferred, less than 40% plant regeneration. In conclusion, an efficient plant regeneration system for *J. curcas* was established through somatic embryogenesis.

Keywords: *Jatropha curcas* , cotyledons, callus , somatic embryogenesis , plant regeneration.

INTRODUCTION

Jatropha curcas, belonging to Euphorbiaceae family, is a perennial, deciduous and oil-bearing shrub. It is originated from South America and can now be found in subtropical and tropical regions of African countries and Asia including Malaysia. Traditionally, *J. curcas* is mainly grown for making soaps, fencing live animals and reclaiming wasteland (Openshaw, 2000). *J. curcas* is now popularly grown for high content of non-edible oil in the seeds that is suitable for biofuel production (Chhetri et al., 2008). Attributed to its incredible value which is profusely acknowledged, large cultivation of *J. curcas* is inevitable at large marginal plantations in worldwide. However, conventional propagation is often limited to be used to mass propagate the desired elite plants to meet the high demand of planting materials. Hence, micropropagation could provide an alternative way for mass propagation. Somatic embryogenesis, a powerful micropropagation tool, has been widely applied to many plant species for mass propagation of planting materials. It had been applied to many important economical crops, such as rice (Sahrawat and Chand, 2001), wheat (Filippov et al., 2006), potato (JayaSree et al., 2001), oil palm (Rajesh et al., 2003), sesame (Mary and Jayabalan, 1997), to ensure the continuous supply of plants for plantations. For *J. curcas*, reported somatic embryogenesis studies are still limited although plant regeneration through organogenesis using different explants such as leaf-discs (Deore and Johnson 2008), axillary nodes (Sujatha et al. 2005) and shoot tips (Rajore and Batra 2005) were reported. Hence, it is important to study and develop an efficient somatic embryo regeneration protocol for efficient propagation and genetic manipulation programmes in future.

Different plant species require different *in vitro* cultural conditions for initiation of somatic embryogenesis. Studies of auxins and cytokinins separately or in their

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Abbreviations: MS, Murashige and Skoog; **WPM,** woody plant medium; **BAP,** N-6-benzylaminopurine; **GA_3,** gibberellic acid; **ABA,** abscisic acid; **PEG 6000**, polyethylene glycol 6000.

combinations to initiate somatic embryogenesis such as in wheat (Filippov et al., 2006), cassava (Ma, 1998) and sesame (Mary and Jayabalan, 1997) were reported. As for *J. curcas*, the leaf explants were used to induce somatic embryos using the medium containing cytokinin (Jha et al., 2007). Despite this, it is still necessary to investigate plant regeneration system of *J. curcas* to improve the somatic embryo plant regeneration efficiency. In addition, plant tissue culture is genotype dependent and the effect of phytohormones on somatic embryogenesis varies depending on the type of explants investigated.

Low frequency of plant regeneration from somatic embryos is a concern in somatic embryogenesis (Maruyama et al., 2005). In most of the studies, plant recovery from somatic embryos faced problems such as abnormal embryo formation (Singh and Hazra, 2009), low embryo maturation (Sunderlikova and Wilhelm, 2002) and precocious germination of somatic embryos (Garcia-Martin et al., 2001). Different improvement strategies were attempted by researches in order to enhance the recovery of plants from somatic embryos by using exogenous phytohormones (Sunderlikova and Wilhelm, 2002) or osmoticums (Stasolla and Yeung, 2003; Yildirim et al.; 2006; Rai et al., 2007). In this study, we reported the induction of embryogenic callus, multiplication of somatic embryos on the suitable medium and approaches to enhance somatic embryo regeneration system.

MATERIALS AND METHODS

Plant materials and seed sterilisation

Mature seeds were dehulled and used. Seeds were dehulled and washed with 30% (v/v) Clorox® for 15 min and followed by another washing with 20% (v/v) Clorox® for 10 min. Two drops of Tween 20 were added during each washing and the tissues were rinsed twice with sterile distilled- water for 2 min after each washing. After washing, the dehulled seeds were immersed in absolute ethanol for 1 min and then rinsed twice with sterile distilled- water for 2 min. Cotyledon tissues were cut into 3 x 3 mm in size and cultured on the medium.

Medium preparation and culture conditions

Murashige and Skoog medium (Murashige and Skoog, 1962) and woody plant medium (Lloyd and McCown 1980) were used in this study. All media were added with 3% (w/v) sucrose and 0.28% (w/v) gelrite. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C and 15 psi for 15 min. Abscisic acid (ABA) and gibberillic acid (GA_3) were filter-sterilised prior to use. All cultures were kept at 25 ± 1°C with 16 h photoperiod (1000 lux) and 8 h darkness.

Initiation of callus for somatic embryo induction

Sterilised cotyledon explants were cultured on MS medium supplemented with single auxin. Auxins investigated in this study were 1-naphthylacetic acid (NAA), 3-indoleacetic acid (IAA), 3-indolebutyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), 4-amino-3,5,6-trichloropicolinic acid (picloram) and 3,6-dichloroanisic acid (dicamba) at various concentrations (0.4, 0.8, 1.2 and 1.6 mg l^{-1}). The pyhtohormone- free MS medium was used as control. Triplicate with 15 explants for each treatment were used and this study was repeated twice. The percentage of callus induction was recorded after four weeks.

Proliferation of somatic embryos

Calli induced from cotyledon explants in the MS medium containing 0.8 mg l^{-1} dicamba turned embryogenic after subculturing the newly-emerged callus into the same medium. These embryogenic calli were used in this study. Approximately 0.2 g of embryogenic calli were weighed and cultured on the phytohormone-free half-strength and full- strength MS and woody plant medium (WMP). Triplicate with a total of nine clumps of the weighed embryogenic calli were used for each treatment. The induction medium (MS containing 0.8 mg l^{-1} dicamba) was used as control. Somatic embryos at different developmental stages were grouped, quantified and recorded based on their morphologies after two weeks of culture and the study was repeated twice.

Plant regeneration from somatic embryos

Two approaches were used in this study. For the first approach, somatic embryos at late cotyledonary stage were cultured on the MS medium supplemented with polyethylene glycol 6000 (PEG 6000) at different concentrations, 2, 4, 6 and 8% (w/v). Besides, ABA at various concentrations, 0.6, 1.2, 1.8 and 2.4 mg l^{-1}, were also investigated. The percentage of embryo maturation (leaflets greening) was recorded after two weeks and the matured somatic embryos were transferred to phytohormone- free MS medium for plant regeneration. Percentage of plant regeneration (somatic embryos with elongated shoots and root formation) was recorded after two weeks. Triplicate with a total of fifteen somatic embryos were used for each treatment and the study was repeated twice. Somatic embryos without treated with ABA and PEG 6000 were used as control.

For second approach, 6-benzylaminopurine (BAP) and GA_3 were used to investigate somatic embryo regeneration. Somatic embryos at late cotyledonary stage were cultured on MS medium containing BAP at various concentrations, 0.1, 0.2, 0.3 and 0.4 mg l^{-1}. For GA_3, the concentrations investigated were 0.2, 0.4, 0.6 and 0.8 mg l^{-1}. The percentage of plant regeneration (somatic embryos with elongated shoots and root formation) was recorded after two weeks. Triplicate with 15 embryos for each replicate were used and the study was repeated twice.

Histological analyses

Embryogenic calli and different stages of somatic embryos were fixed in fixative solution, formalin-acetic acid-alcohol (FAA) in ratio of 1:1:18, for 24 h. The tissues were then dehydrated in a gradual series of tertiary-butyl alcohol (TBA) solutions. After that, specimens were embedded in paraffin wax and sectioned to 8 to 10 µm thicknesses using a microtome (Leica RM 2235, Germany), mounted on the slides and stained with 0.5% (w/v) of haematoxylin and eosin. The stained samples were observed under light microscope (Leica CME, Germany).

Statistical analyses

Data recorded were analyzed using one-way analysis of variance

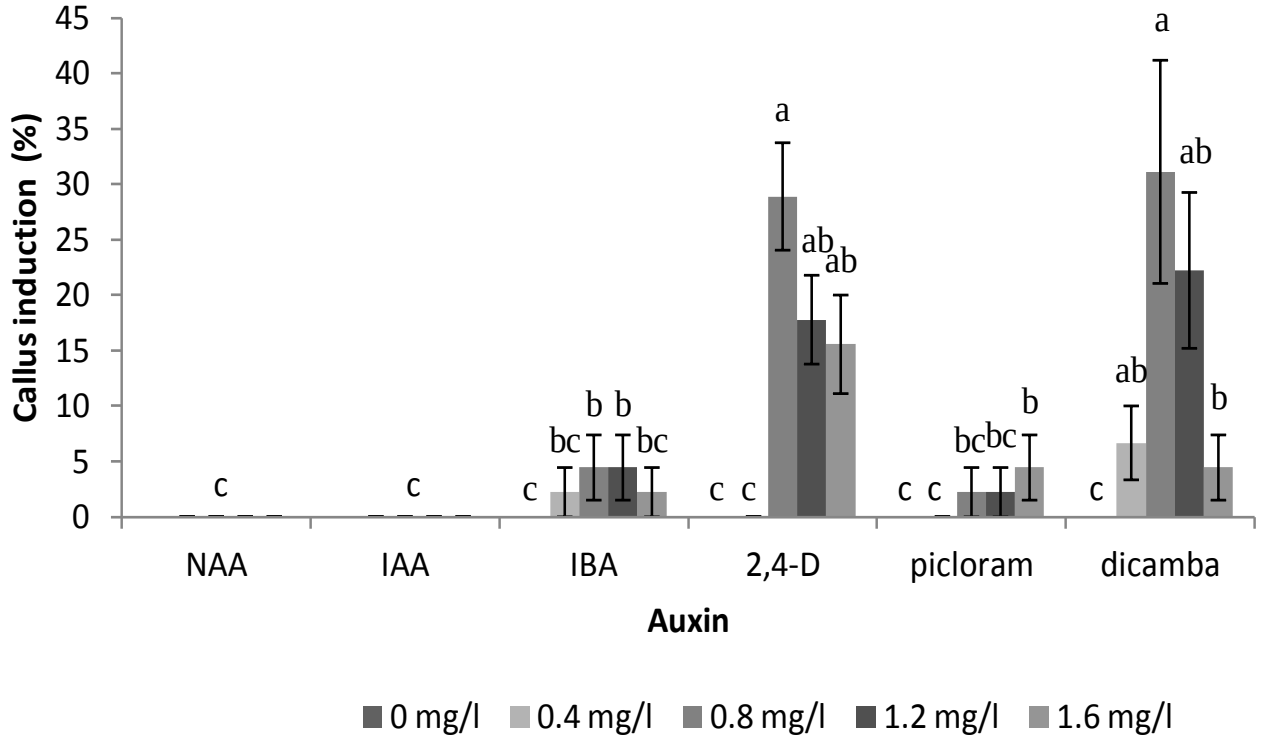


Figure 1. Effects of single auxins on callus induction using cotyledon explants. Mean values followed by same letters are not significantly different according to Tukey's HSD test at *P* = 0.05. Bars indicate standard errors. HSD, Honestly significant difference.

(ANOVA) in SPSS 15.0 software (SPSS Inc. USA). Significant differences between groups were compared using Tukey's honestly significant difference (HSD) test at significance level of 0.05.

RESULTS

Induction of callus and somatic embryos

The response of cotyledon explants to various auxins on callus formation was different. Results showed that single auxin, IBA, 2, 4- D, picloram and dicamba, were suitable for callus induction from cotyledon explants. The highest callus induction, 31.1%, was obtained from the MS medium containing 0.8 mg l^{-1} dicamba and followed by 28.9% from the MS medium containing 0.8 mg l^{-1} 2,4- D (Figure 1). Significant decrement of callus induction was observed when higher concentration (1.6 mg l^{-1}) of dicamba and lower concentration (0.4 mg l^{-1}) of 2,4-D were used. Besides, no callus was induced when MS media containing NAA and IAA and the control medium were used.

Among all callus induction media investigated, the MS medium containing 0.8 mg l^{-1} dicamba was embryogenic. The embryogenic callus was obtained from the newly-formed callus (Figure 2A) emerged from the old callus cultures on the MS medium containing 0.8 mg l^{-1} dicamba after subculturing. The embryogenic callus was friable, creamy and pale yellow in colour (Figure 2B) and able to be multiplied by subculturing at one month interval. Somatic embryos (Figure 2C) were formed and observed from the embryogenic calli. From histological examinations, proembryogenic mass (PEM) was observed on the embryogenic tissues (Figure 2D) indicating the differentiation of callus tissues forming somatic embryos.

Proliferation of somatic embryos

The somatic embryo induction medium was not suitable for proliferation of somatic embryos as shown in Figure 3 in which the number of somatic embryos for each stage obtained was significantly lower than all other media investigated. In contrast, media without addition of dicamba were found to be suitable for proliferation of somatic embryos. However, the number of somatic embryos (at different developmental stages) obtained varied when cultured on different media (Figure 3). Among all proliferation media investigated, full-strength WPM was more suitable for proliferation of somatic embryos. The most abundant globular embryos (331.12 per gram of embryogenic callus), heart-shape (268.33 per gram of embryogenic callus), torpedo (198.13 per gram of embryogenic callus) and cotyledonary embryos

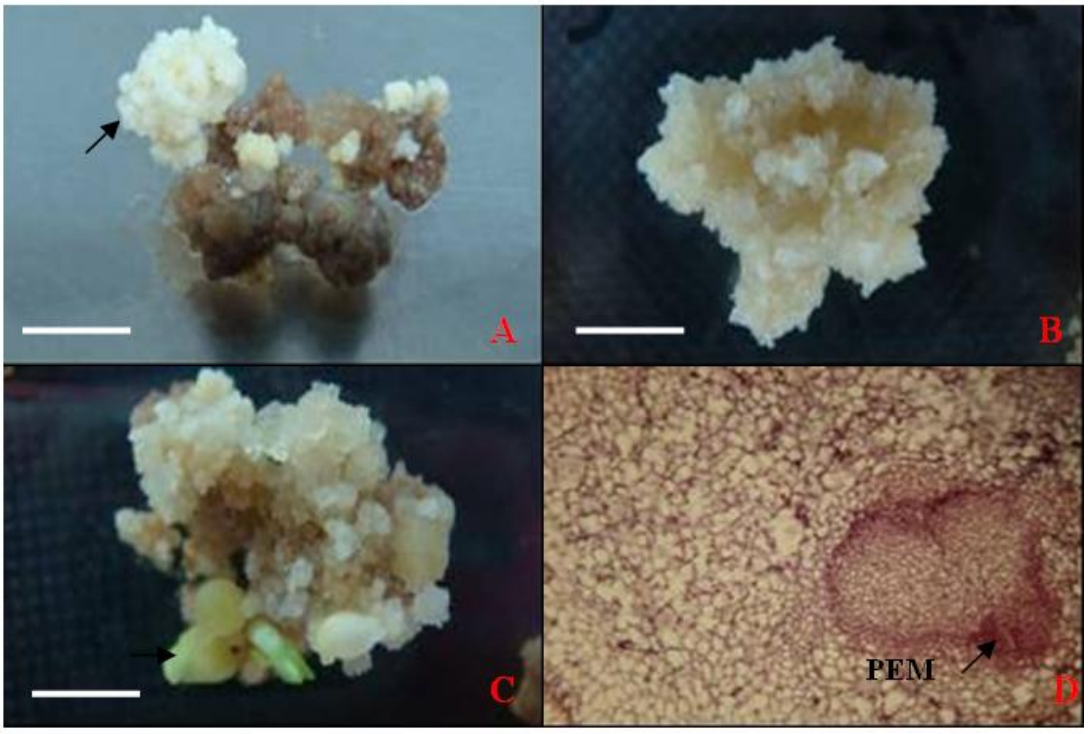


Figure 2. Induction of somatic embryos from cotyledon explants. A, The newly-formed embryogenic-like calli (arrow) from old callus culture; B, embryogenic-like calli; C, somatic embryos (arrow) obtained from the embryogenic calli; D, histological observation on cross-sectioned embryogenic calli shows the proembryogenic mass (PEM) (arrow). Scale bars = 5 mm.

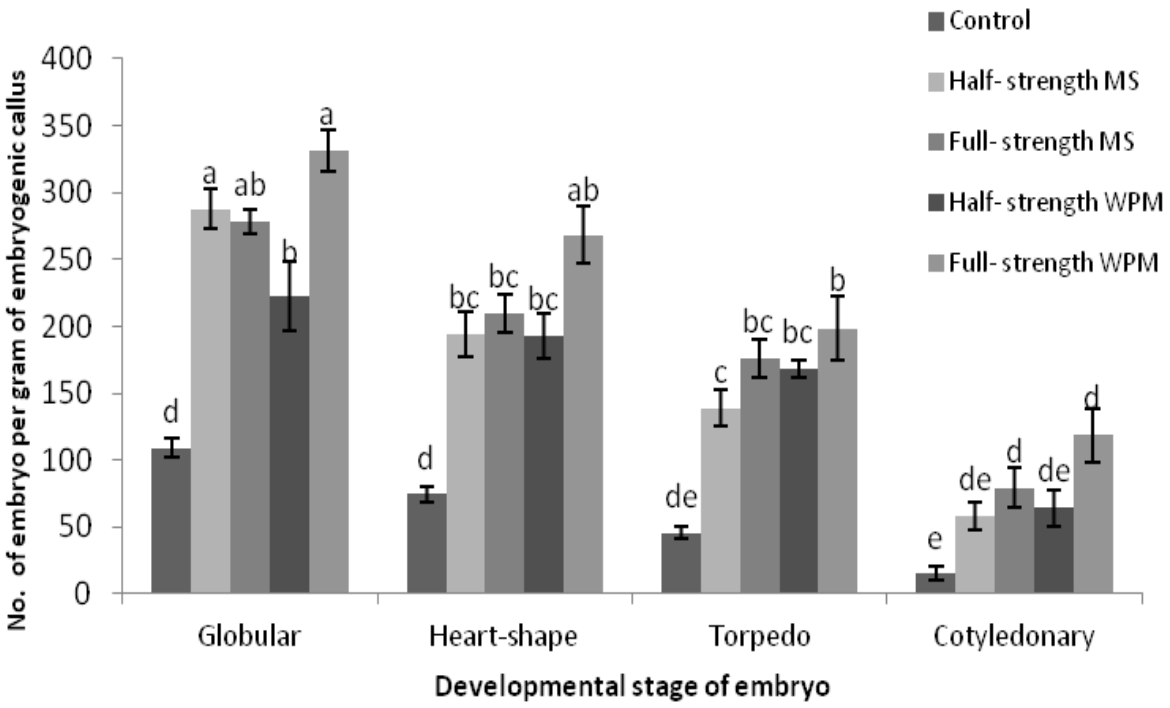


Figure 3. Effects of different media formulations on the proliferation and deveopment of somatic embryos. Mean values followed by same letters are not significantly different according to Tukey's HSD test at $P = 0.05$. Bars indicate standard errors. HSD, Honestly significant difference.

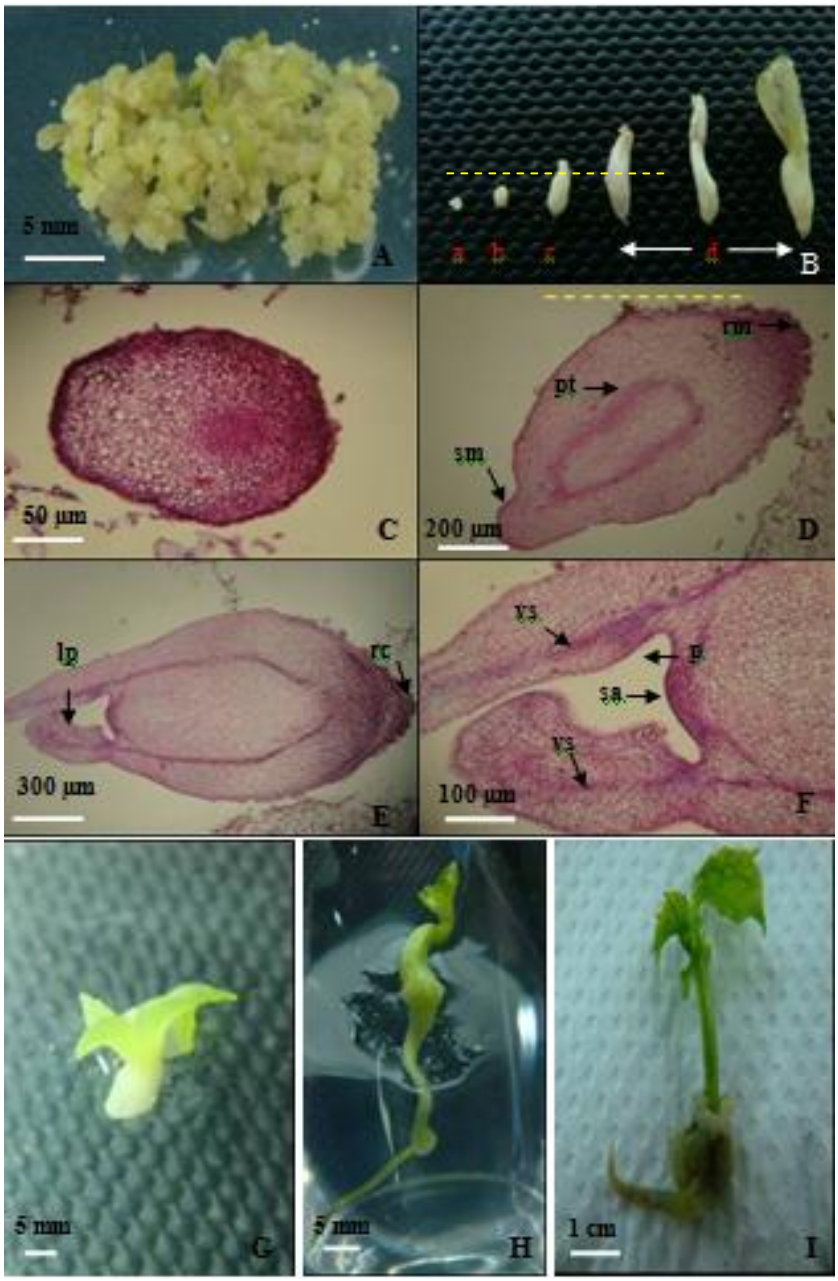


Figure 4. Different stages of somatic embryo plant regeneration. A, Somatic embryos; B, different developmental stages (a = globular, b = heart-shaped, c = torpedo, d = cotyledonary stage) of somatic embryos; C, cross-section of a globular embryo; D, longitudinal-sectioned of a torpedo embryo show the formation of shoot meristerm (sm), root meristem (rm) and procambium tissue (pt); E, leaf primidia (lp) and root cap (rc) derive from root meristerm (rm) are later observed on the longitudinal-sectioned cotyledonary embryo; F, the longitudinal-sectioned cotyledonary embryo also shows the shoot apex (sa) protoderms (p) and vascular strands (vs); G, a matured embryo; H, germination of a somatic embryo; I, a regenerated plantlet.

(118.90 per gram of embryogenic callus) were obtained using full-strength WPM. Globular embryos were the most abundant stage of embryos obtained and followed by heart-shape, torpedo embryos and cotyledonary embryos.

On all the proliferation media investigated, repetitive embryogenesis was observed (Figure 4A) and it was a feature of spontaneous proliferation of somatic embryos.

Various developmental stages of somatic embryos (Figure 4B) were obtained and they were distinguished based on morphologies after two weeks on proliferation media. Histological analysis showed the intense actively dividing cells appeared during the globular stage (Figure 4C). For the torpedo embryos, differentiated tissues such as shoot and root meristerms and procambium tissues were observed (Figure 4D). At late cotyledonary stage (Figures 4E to F), structures such as root cap, leaf primodia, shoot apex, vascular strand and protoderms were observed.

Plant regeneration study

Due to low frequency of somatic embryos which were regenerated into plantlets on all the proliferation media investigated, somatic embryos were matured in the medium containing either ABA or PEG 6000 prior to plant regeneration. Our study found that matured somatic embryos (Figure 4G) were obtained after treated with ABA and PEG 6000. In general, matured somatic embryos obtained from different maturation media were able to germinate into plantlets (Figure 4I) on the phytohormone- free MS medium after two weeks. High embryo maturation, 77.8 and 62.2%, were obtained on the MS medium containing 0.6 mg l^{-1} ABA (Figure 5A) and 4% PEG 6000 (Figure 5B), respectively. In contrast, embryo maturation was found to be significantly low in the control medium. However, efficacy of obtaining matured somatic embryos was not significantly different among the concentrations investigated in this study for both ABA and PEG 6000.

For plant regeneration, our results showed that the highest plant regeneration, 35.6% (Figure 5A), was obtained from the MS medium containing 0.6 mg l^{-1} ABA. On the other hand, the medium containing PEG 6000 achieved the highest plant regeneration (28.9%) at the concentration of 6% (Figure 5B). No significant different in plant regeneration was observed among the concentrations investigated for both ABA and PEG 6000 treated somatic embryos. However, plant regeneration frequency was significantly higher than that of the untreated somatic embryos from the control medium. In general, the matured somatic embryos obtained from different maturation media were able to germinate into plantlets after transferring to phytohormone- free MS medium.

Besides, the induced somatic embryos (at cotyledonary stage) were able to directly regenerate into plantlets after two weeks cultured on the MS media containing BAP and GA_3. The results show that the highest plant regeneration, 83.3%, was obtained from the MS medium containing 0.3 mg l^{-1} BAP while 73.3% plant regeneration was obtained from the MS medium containing 0.4 mg l^{-1} GA_3 (Figure 6). No significant different on the plant regeneration frequency among the concentrations investigated for both BAP and GA_3 was observed. Somatic embryo plant regeneration was significantly low in the control medium. In general, more than 60% plant regeneration was achieved from the medium containing BAP and GA_3 regardless of the concentration used. After four weeks, the regenerated somatic embryos with newly developed leaves were observed on the MS medium containing BAP or GA_3. Plant regeneration was more efficient when the somatic embryos were directly cultured on the MS medium containing BAP or GA_3 than the somatic embryos that were treated with ABA or PEG 6000. The duration for obtaining regenerated plantlets was also shortened, two weeks was required instead of four weeks by using embryo maturation approach.

DISCUSSION

Induction and proliferation of somatic embryos

A number of approaches had been used to achieve plant regeneration via somatic embryogenesis in many plant species. Jha et al. (2007) reported somatic embryogenesis of *J. curcas* from leaf tissues using single cytokinin, kinetin. In our study, somatic embryos of *J. curcas* were obtained from cotyledon-derived calli in the medium containing dicamba. In general, young tissues such as cotyledon tissues possess higher regeneration ability (Hoque and Mansfield, 2004; Prakash and Gurumurthi, 2010) and are suitable to be used as explants for initiation of embryogenic callus. Cotyledon tissues had also been successfully used for induction of somatic embryogenesis for many woody plant species such as *Dalbergia sissoo* Roxb (Singh and Chand, 2003), *Azadirachta indica* (Gairi and Rashid, 2005) and *Protea cynaroides* (Wu et al., 2007).

In this study, embryogenic callus was initiated after subculturing the induced calli on the induction medium containing dicamba. According to Michaux-Ferriere and Carron (1989), a suitable subculturing time is important to initiate somatic embryogenesis from the callus. Dicamba was also used to induce somatic embryogenesis of several *Triticum* sp. (Filippov et al., 2006). Other than dicamba, single auxins such as NAA, IAA, IBA, 2, 4- D and picloram were also able to induce somatic embryogenesis of *Myrtus communis* (Canhoto et al., 1999) and *Sesamum indicum* (Mary and Jayabalan, 1997). In contrast to our study, Kalimuthu et al. (2007) reported a direct embryogenesis of *J. curcas* using cotyledon tissues in the medium containing BAP; however, plantlet regeneration and somatic embryo multiplication were not reported in their studies.

The role of auxin is subjected to change after embryogenesis is induced. Less auxin is required as somatic embryos begin to synthesize their own auxins for proliferation (Zimmerman, 1993). Hence, the media without phytohormones were used for proliferation of

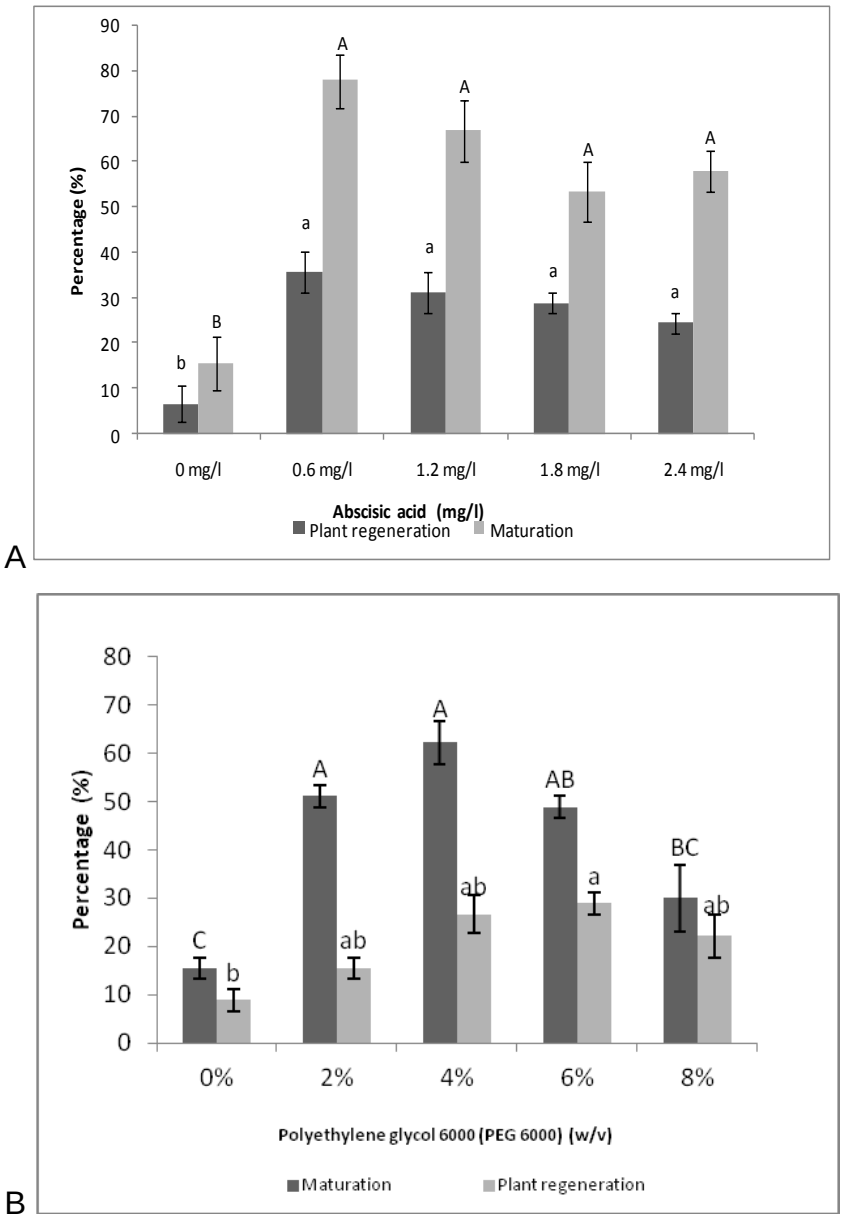
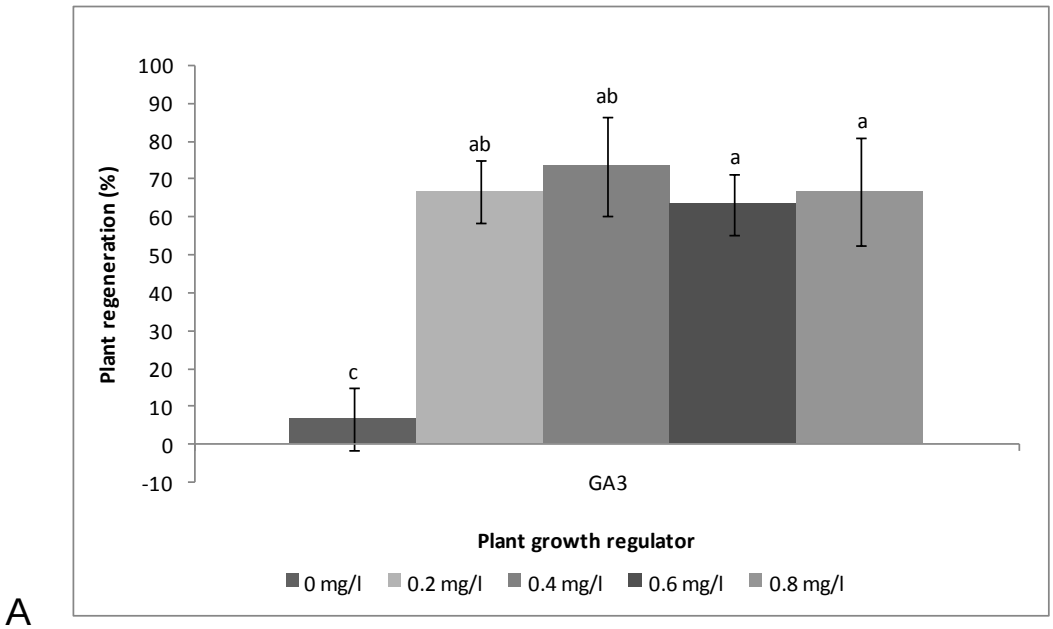


Figure 5. Effects of (A) abscisic acid and (B) polyethylene glycol 6000 on embryo maturation and plant regeneration. Mean values between groups followed by same letters are not significantly different according to Tukey's HSD test at P = 0.05. Bars indicate standard errors. HSD, Honestly significant difference.

somatic embryos while reducing the chance of obtaining somaclonal variation of somatic embryos (Pinto et al., 2008). Similarly, the phytohormone- free medium was used in proliferation of somatic embryos in this study. In our study, proliferation of embryos was more efficient on the full-strength WPM. This probably attributed to the salt content of WPM used was suitable for proliferation and development of different stages of somatic embryos. Similar studies from Fisichella et al. (2000) and Kintzios et al. (2001) also showed that different salt contents supplemented into the medium would influence the development and proliferation of somatic embryos.

Plant regeneration from somatic embryos

Embryo maturation is an essential phase during embryogenesis in which the synthesis of storage reserves such as starch, lipids, and proteins occurred (Sunderlikova and Wilhelm, 2002). Jha et al. (2007) used the medium containing combinations of adenine sulphate, kinetin and IBA to mature *J. curcas* somatic embryos. However, less



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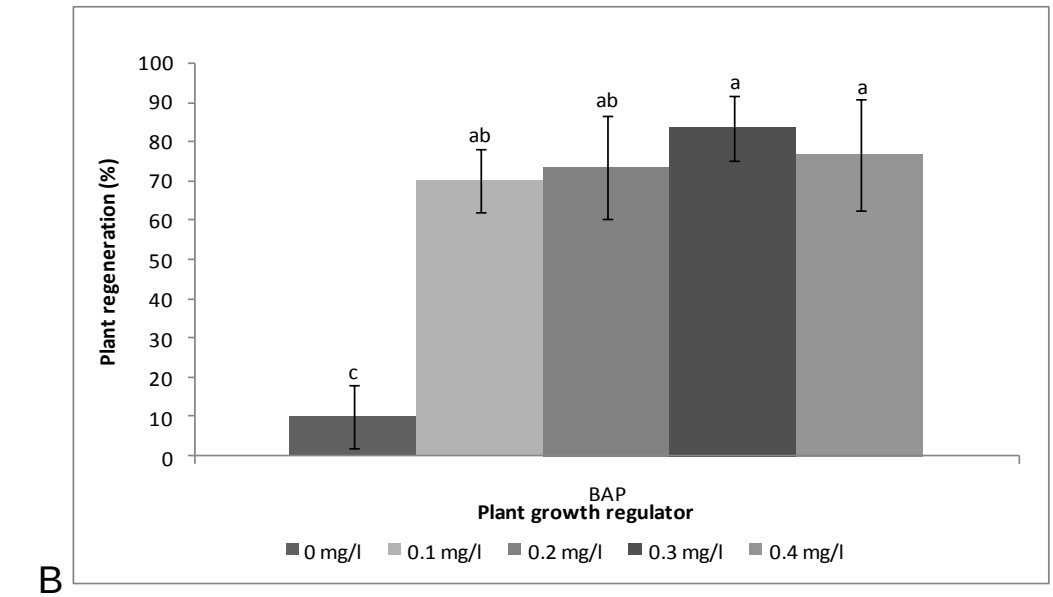

B

Figure 6. Effects of (A) GA_3 and (B) BAP on direct plant regeneration from somatic embryos. Mean values between groups followed by same letters are not significantly different according to Tukey's HSD test at P = 0.05. Bars indicate standard errors. HSD, Honestly significant difference; BAP, N-6-benzylaminopurine; GA_3, gibberellic acid.

than 50% matured somatic embryos were obtained, the plantlets recovery from the matured somatic embryos was low (20%) and the duration required for plant regeneration was about six weeks in their study. In contrast, a higher percentage of matured somatic embryos was obtained in this study, 77.8% from the medium containing 0.6 mg l^{-1} ABA. Plant regeneration from somatic embryos was also higher after pre-culturing them on the MS medium containing 0.6 mg l^{-1} ABA (35.6%). The duration required for the cotyledonary stage somatic embryos regenerated into plantlets was about four weeks. Besides, plant regeneration was improved by directly culturing the somatic embryos on the MS medium containing 0.3 mg l^{-1} BAP (83.3%) and 0.4 mg l^{-1} GA_3 (73.3%). The beneficial effect of BAP was further evident when somatic embryogenesis of *J. curcas* was able to directly achieve (Kalimuthu et al. 2007). The plant regeneration duration required was shortened to two weeks by excluding maturation phase using ABA or PEG in this study.

Thus, BAP and GA_3 were more suitable for direct plant regeneration from somatic embryos in our study. In contrast, Yildirim et al. (2006) and Zhang et al. (2007) reported plant regeneration from somatic embryos was enhanced by adding ABA or PEG or combination of ABA and PEG. Efficiency of somatic embryos regeneration could be due to the effects of different genotypes used as *J. curcas* might be very genotype dependent and somatic embryos were originated from different types of explants. In other studies, BAP and GA_3 were able to directly regenerate somatic embryos by either supplementing individually or in combination into the medium for somatic embryo germination of *Centella asiatica* (Paramageetham et al., 2004), *Catharanthus roseus* (Junaid et al., 2006) and *Protea cynaroides* (Wu et al., 2007).

In brief, this study described the complete regeneration process of *J. curcas* via somatic embryogenesis. Our results suggest that somatic embryo maturation phase was not necesssary if somatic embryo regeneration was carried out using the media containing BAP and GA_3.

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