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PRIMARY AND SECONDARY SOMATIC EMBRYOGENESIS IN *JATROPHA CURCAS* L. FROM LEAF TRANSVERSE THIN CELL LAYERS

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SUMMARY

An efficient method for plant regeneration in *Jatropha curcas* L. via primary and secondary somatic embryogenesis culture from *ex vitro* leaves of 6-month-old plants was presented in this study. Leaves were cut into transverse thin cell layers (tTCLs) and cultured on MS medium supplemented with kinetin (KIN) at 0.5, 1.0, 1.5, and 2.0 mg/l in combination with indole-3-butyric acid (IBA) at 0.1, 0.5, and 1.0 mg/l or 2,4-dichlorophenoxyacetic acid (2,4-D) at 1.0, 1.5 and 2.0 mg/l . The highest embryogenic callus formation rate (89.3%) was obtained on medium supplemented with 1.0 mg/l KIN and 1.5 mg/l 2,4-D. The calli were selected for the study of primary somatic embryogenesis on MS medium containing 2,4-D (0.01, 0.03, 0.05, and 0.07 mg/l) or KIN (0.5, 1.0, 1.5, and 2.0 mg/l). The highest primary somatic embryos formation rate (76.67%) was achieved on MS medium supplemented with 1.0 mg/l KIN. The primary embryos were cultured on medium supplemented with KIN (0.1, 0.5, 1.0, 1.5, and 2.0 mg/l) combined with 0.2 mg/l indole-3-butyric acid **(**IBA) or 0.05 mg/l 2,4-D. The combination of 1.5 mg/l KIN and 0.05 mg/l 2,4-D was suitable for secondary embryos formation. Embryos proliferated rapidly, and the highest number of secondary embryos (77.5 embryos) wasobtained from a single primary embryos inoculated. Results also showed that the addition of proline (0.75 g/l) or spermidine (0.15 mM) to the culture medium increased the number of secondary embryos considerably. The fully developed plantlets exhibiting healthy roots and shoots were obtained when somatic embryos were sub-cultured onto B5 medium containing 1.5 mg/l IBA.

Keywords: *Jatropha curcas* L.*, Proline, somatic embryogenesis, secondary somatic embryogenesis, spermidine*

INTRODUCTION

Somatic embryogenesis is the most efficient method in *in vitro* propagation of plants. Somatic embryogenesis was first induced in suspension and callus cultures of carrot (Steward *et al*., 1958; Reinert, 1959). Since then, this species has been widely used to study different aspects of somatic embryogenesis *in vitro* (Molle *et al*., 1993). Somatic embryogenesis is influenced by several factors in culture medium including plant growth regulators (PGRs) (Steward *et al*., 1964), amino acids (such as proline, serine and threonine) (Thorpe, 1993), polyamines (such as spermidine and spermine) (Steiner *et al*., 2007) and carbohydrate sources (Hong *et al*., 2008). This method has been used for multiplication of plants with high economic values including palm oil (Rajesh *et al*., 2003), *Citrus* sp. (Gavish *et al*., 1991, 1992), *Coffea* sp. (Monaco *et al*., 1977), *Medicago* sp. (McKersie *et al*., 1993), *Zea mays* (Emons, 1994), *etc.*

Secondary embryogenesis is more effective than primary embryogenesis in plant propagation. Secondary somatic embryos are rapidly produced through the process of secondary somatic embryogenesis from primary somatic embryos (Singh, Chaturvedi, 2009). The secondary somatic embryogenesis had shown more significant efficiencies than primary somatic embryogenesis including high multiplication rates regardless of the original explant sources and a high degree of uniformity (Singh, Chaturvedi, 2009). Highly effective regeneration systems via secondary somatic

embryogenesis have been reported in many plant species including neem (Singh, Chaturvedi, 2009), soybean (Gyulai *et al*., 1993) and rose (Bao *et al*., 2012).

Jatropha curcas L. (physic nut) is a bush plant belonging to the family Euphorbiaceae, which comprises approximately 8,000 species. *J. curcas* seeds have oil content of about 30-40%, and crude oil from the seeds is processed into biodiesel and many other valuable products such as organic fertilizers, bio-pesticides and pharmaceutical products. About ten years ago, many countries around the world including countries in Asia interested in research and development of *J. curcas* for biodiesel production, particularly the seed quality and cultivation of the first line of the tree to develop raw materials (Rajore, Batra, 2005). The direct seedling method has problems such as poor seed viability, low germination, unstable yield and oil content while plants propagated by cuttings show a lower longevity and possess a lower drought and disease resistance than those propagated by seeds. Alternatively, micropropagation is an efficient approach for rapid multiplication of *J. curcas*, generating seedlings from cultures of different parts of the plant such as axillary buds, shoot tip, node and leaf (Sujatha, Mukta, 1996; Sardana *et al*., 1998; Rajore, Batra, 2005; Sujatha *et al*., 2005). Somatic embryogenesis has also been applied successfully on *J. curcas* (Jha *et al*., 2007; Siang *et al*., 2012). However, no study about the increase in frequency of primary and secondary somatic embryogenesis has been published. Here, we reported a specific qualitative and quantitative investigation of primary and secondary somatic embryogenesis to improve the micropropagation in *J. curcas*.

MATERIALS AND METHODS

Plant Material

The second pair of leaves from the top of six-month-old *J. curcas* plants were collected and washed under running tap water for 30 min, then surface sterilized in 70% (v/v) alcohol for 30s followed by three to four rinses with sterilized distilled water. In a laminar air flow chamber, leaves were sterilized in NaOCl solution (50%, v/v) with two to three drops of Tween-20 for each 100 ml solution for 10 min and rinsed four to five times in sterilized distilled water.

Culture Media

Explants were cultured on MS basal medium (Murashige, Skoog, 1962) supplemented with 30 g/L sucrose, 8 g/L agar and PGRs at different concentrations. The pH of the medium was adjusted to 5.8 prior to autoclaving for 15 min at 121°C (1 atm).

Callus Formation

Disinfected leaves were cut into tTCLs (0.5 mm in thickness × 10 mm in width) and cultured on MS medium supplemented with kinetin (KIN) at 0.5, 1.0, 1.5 and 2.0 mg/l in combination with indole-3-butyric acid (IBA) at 0.1, 0.5 and 1.0 mg/l or 2,4-dichlorophenoxyacetic acid (2,4-D) at 1.0, 1.5 and 2.0 mg/l. After four weeks of culture, embryogenic calli were selected for study of primary somatic embryogenesis.

Primary Somatic Embryogenesis

Embryogenic callus (soft, friable and light creamish) clumps were cut into 1.0 cm square and transferred to MS medium containing 2,4-D (0.01, 0.03, 0.05 and 0.07 mg/l) or KIN (0.5, 1.0, 1.5 and 2.0 mg/l) in order to obtain somatic embryogenesis.

Secondary Somatic Embryogenesis

Single primary embryos at cotyledonary stage were isolated and cultured on MS medium supplemented with KIN (0.1, 0.5, 1.0, 1.5 and 2.0 mg/l) combined with 0.2 mg/l IBA or 0.05 mg/l 2,4-D.

Single primary embryos at cotyledonary stage were also cultured on MS medium supplemented with PGRs and proline (100, 200, 300, 400 and 500 mg/l) or spermidine (0.1, 0.15, 0.2, 0.25 and 0.3 mM) in order to investigate the influence of these compounds on somatic embryo proliferation.

Plant Regeneration

Secondary somatic embryos with young green cotyledons and the root primordial were cultured on various nutrient media including ½MS (half strength MS medium - half-strength macronutrients, full-strength micronutrients and vitamins), MS, B5 (Gamborg *et al*., 1976), White (White, 1943), WPM (Lloyd, McCown, 1981) without supplement of PGRs. The optimal mineral salt formulation were supplemented with IBA or 1-naphthaleneacetic acid (NAA) (0.5, 1.0, 1.5 and 2.0 mg/l) for plant regeneration.

Culture Conditions

Cultures were maintained at 14h of light per day, and 45±5 µmol.m^{-2}.s^{-1} of photosynthetic photon flux density. Temperature was at 25±2ºC and relative humidity was at 60±5%.

Statistical analysis

All the experiments were triplicated with ten explants per replication. Data were subjected to analysis of variance with mean separation of Duncan's multiple range test (Duncan 1955) using Statgraphics Centurion XV (StatPoint Technologies Inc., Warrenton, VA, USA).

RESULTS AND DISCUSSION

Callus Formation

Jha *et al*. (2007) suggested that PGRs were the main factors to determine the formation of embryogenic calli of *J. curcas*. In this study, leaf tTCLs cultured on basic MS medium supplemented with different concentrations of KIN showed swelling after five to seven days of culture. On PGR-free medium, leaf explants also swelled, and then slowly turned to brownish and necrotized. Table 1 showed that the combinatorial effect of KIN and IBA induced the callus formation, and those calli were compact and bright in color (yellow or green) (Fig. 1a, 1b). The callus formation rates on medium supplemented with KIN and IBA (37.5 to 70.3%) were lower than those on medium supplemented with KIN and 2,4-D (45.2 to 89.3%), in which the soft, friable and yellow calli were observed.

Table 1. Effect of KIN in combination with IBA or 2,4-D on callus formation from *ex vitro* leaves. a, b, c… in the same column show the differences between the means of each of the treatments (Duncan's multiple range test: P<0.05).

PGRs (mg/l)			Callus formation (%)	Morphology of callus
KIN	IBA	2,4-D		
-	-	-	-	Swelling, dark brown
0.5	0.1	-	37.5e (*)	Compact, light yellowish
1.0	0.1	-	40.8de	Compact, light yellowish
1.5	0.1	-	42.9de	Compact, light yellowish
2.0	0.1		36.9e	Compact, light yellowish
0.5	0.5	-	62.1c	Compact, light yellowish
1.0	0.5	-	70.3bc	Compact, creamish
1.5	0.5	-	65.4c	Compact, light greenish
2.0	0.5	-	43.8d	Compact, light greenish
0.5	1.0	-	56.1cd	Compact, light greenish
1.0	1.0	-	60.3c	Compact, light greenish
1.5	1.0	-	61.6c	Compact, dark brown
2.0	1.0	-	49.8d	Compact, dark brown
0.5	-	1.0	45.2d	Soft, friable, creamish
1.0	-	1.0	46.7d	Soft, friable, creamish
1.5	-	1.0	46.9d	Soft, friable, light creamish
2.0	-	1.0	44.3d	Soft, friable, light creamish
0.5	-	1.5	72.4bc	Soft, friable, light creamish
1.0	-	1.5	89.3a	Soft, friable, light creamish
1.5	-	1.5	75.1b	Soft, friable, creamish, nodular
2.0	-	1.5	53.1d	Soft, friable, creamish, nodular
0.5	-	2.0	76.1b	Soft, flabby, white
1.0	-	2.0	80.3b	Soft, flabby, white
1.5	-	2.0	63.7c	Soft, flabby, white
2.0	-	2.0	56.2cd	Soft, flabby, white

Jha *et al*. (2007) investigated the embryogenic callus formation in *J. curcas* from leaf explant on medium MS containing of KIN or BA at various concentrations. Their results showed that KIN at 2.0 mg/l induced embryogenic calli, which had many small cream nodules after four weeks of culture. Besides, Siang *et al*. (2012) obtained cotyledon-derived embryogenic calli of *J. curcas* on medium containing dicamba. In our study, after the same culture period, callus formation rate reached the highest rate (89.3%) on medium supplemented with 1.0 mg/l KIN and 1.5 mg/l 2,4-D. Calli were soft and friable with light cream color and showed the ability to induce somatic embryogenesis. Smith and Krikorian (1989) found that during the process of somatic embryogenesis, the integrity of the tissue was broken down. The separation of cells made soft and friable calli, and the calli or cutin layer was formed (Merkle *et al*., 1995). Those calli had the ability to transform into somatic embryos.

Primary Somatic Embryogenesis

Previous studies have supported the hypothesis that PGRs and other methods were used to induce embryogenesis by changing the polarization of cells and promote the asymmetric division of cells (de Jong *et al*., 1993). Among growth regulators used, 2,4-D and KIN are usually supplemented into medium to induce somatic embryogenesis. In the present work, the highest rate of embryogenesis (76.67%), number of embryos (39.70 embryos), and fresh weight of embryos clusters (0.058g) were achieved on medium supplemented with only 1.0 mg/l KIN (Table 2, Fig. 1c). Different results were reported by Jha *et al*. (2007) when investigating the effects of combinations of auxin and cytokinin on the embryogenesis from leaf-derived calli of *J. curcas*. The highest of embryogenesis rate and the number of embryos were obtained on medium supplemented with 0.5 mg/l KIN and 0.25 mg/l IBA.

Table 2. Effect of 2,4-D and KIN on somatic embryogenesis from callus. a, b, c… in the same column show the differences between the means of each of the treatments (Duncan's multiple range test: P<0.05).

PGRs (mg/l)		Embryogenesis (%)	Number of embryos/explant	Fresh weight (g)
2,4-D	KIN			
-	-	0.00e	0.00e	0.00g
0.01	-	30.00e	8.23e	0.010f
0.03	-	43.33d	11.17e	0.013ef
0.05	-	63.33b	20.03c	0.031c
0.07	-	46.67cd	12.20de	0.014e
-	0.5	40.00de	16.70cd	0.019d
-	1.0	76.67a	39.70a	0.058a
-	1.5	56.67bc	26.37b	0.045b
-	2.0	50.00bc	21.30c	0.034c

Cytokinin is necessary for somatic embryogenesis at different levels depending on specific plant species. KIN has been shown to work well the first time to form somatic embryos in some woody species (Dunstan *et al*., 1995). The use of individual cytokinin to induce somatic embryos from immature zygote embryos has also been successful in some species of both angiosperms and gymnosperms. Cytokinin stimulates cell division without being constrained by the surrounding cells. For instance, after embryogenic calli of *Parkia biglobosa* were formed, they were transferred to medium without auxin to develop into somatic embryos (Amoo *et al*., 2004). In addition, cytokinins also stimulate the formation of somatic embryos from non-embryogenic calli in other species such as peanut (Gill, Saxena, 1992). Kalimuthu *et al*. (2007) concluded that the embryos of *J. curcas* were also scored on medium containing only an individual cytokinin such as 6-benzylaminopurine (BA).

Secondary Somatic Embryogenesis

Somatic embryogenesis which has been established in tissue culture can be maintained for a long time by repeating cycles of secondary somatic embryogenesis (Shi *et al*., 2010). Secondary embryos tend to be derived from epithelial cells (Raemakers *et al*., 1995). The morphology of secondary embryos is similar to those of primary embryos and zygote embryos in which they go

through globular, heart, torpedo and cotyledon stage (Zimmerman, 1993). Primary somatic embryos of *J. curcas* were cultured on MS medium containing KIN combined with IBA or 2,4-D to investigate somatic embryo proliferation through secondary somatic embryogenesis. After six weeks of culture, secondary somatic embryos were formed in all treatments (except the control treatment-without PGRs). Secondary somatic embryos formation rate on medium containing KIN combined with IBA was lower than those on medium containing KIN and 2,4-D. The highest embryogenesis rate (93.3%), total number of embryos formed (77.5 embryos), fresh weight of embryos (0.087 g), and numbers of embryos in all stages were obtained on medium supplemented with 1.5 mg/l KIN and 0.05 mg/l 2,4-D (Table 3, Fig. 1d). This result gave an efficient approach to rapidly multiplication of somatic embryos from primary embryos in *J. curcas*.

Table 3. Effect of KIN in combination with IBA or 2,4-D on secondary somatic embryogenesis. a, b, c… in the same column show the differences between the means of each of the treatments (Duncan's multiple range test: P<0.05).

PGRs (mg/l)			Embryogenesis (%)	Number of embryos/ explant	Globular stage	Heart stage	Torpedo stage	Cotyledon stage	Fresh weight (g)
KIN	IBA	2,4-D							
-	-	-	0.0e	0.0g	0.0g	0.0g	0.0e	0.0e	0.0g
0.1	0.2	-	36.7d	21.8f	13.9f	5.3f	2.37d	0.03e	0.027f
0.5	0.2	-	43.3d	27.3f	16.2f	6.8f	4.07c	0.17de	0.033f
1.0	0.2	-	60.0c	48.7cde	30.6cde	10.8de	6.97b	0.27cde	0.055de
1.5	0.2	-	76.7b	52.2cde	32.5cde	11.7cde	7.60b	0.47c	0.06cd
2.0	0.2	-	60.0c	42.5e	25.9e	9.7e	6.57b	0.40cd	0.049e
0.1	-	0.05	63.3c	46.2de	29.3de	10.4de	7.13b	0.33cd	0.054de
0.5	-	0.05	70.0bc	54.1bcd	34.1cd	12.4bcd	7.23b	0.40cd	0.062bcd
1.0	-	0.05	76.7b	58.8bc	37.4bc	13.6bc	7.40b	0.43cd	0.066bc
1.5	-	0.05	93.3a	77.5a	49.8a	17.03a	9.53a	1.17a	0.087a
2.0	-	0.05	80.0b	63.4b	41.0b	14.3b	7.10b	0.87b	0.072b

Secondary somatic embryogenesis was not appeared when primary embryos were cultured on PGR-free medium (Singh, Chaturvedi, 2009). Kumar and Kumari (2010) reported rapid plant regeneration system through primary and secondary embryogenesis of *Carthamus tinctorius* L. The secondary embryos formed directly from the primary embryos at cotyledons stage on medium supplemented with BA and NAA. Secondary embryogenesis has great potential for large-scale micro-propagation, particularly for woody plants which have long regeneration cycle and low frequency of somatic embryogenesis. Recently, Shi *et al*. (2010) studied the formation of secondary embryos in camphor tree (*Cinnamomum camphora* L.) and successfully developed the secondary embryos from the primary embryos at cotyledon stage on the medium supplemented with ABA.

Somatic Embryo Proliferation

Proline is the amino acid best known in plant tissue culture for its effects on embryogenesis stimulation (Shimizu *et al*., 1997). In this experiment, when primary embryos were cultured on MS medium supplemented with 1.5 mg/l KIN, 0.05 mg/l 2,4-D and proline at concentrations from 100 to 500 mg/l, there is no significant increase in the secondary embryogenesis rate was recorded. However, the result showed that the highest number of embryos (111.9 embryos per explant) and the fresh weight of embryos (0.125g) were obtained at 300 mg/l, and the maximum number of embryos in all stages was also produced at 300 mg/l proline (Table 4, Fig. 1e, 1f).

Proline is used separately or in combination with

other amino acids, and it may stimulate the somatic embryogenesis in different plant types. For example, proline and serine stimulate the somatic embryogenesis in orchard grass (Trigianno, Conger, 1987), proline alone stimulates somatic embryogenesis in carrot (Nuti-Ronchi *et al*., 1984), and the quality of alfalfa embryos is improved on medium supplemented with 50-300 mM proline. Santos *et al*. (1996) found that the addition of proline provides significant effects on total protein amount of embryogenic calli. They suggested that the presence of proline in the culture medium responded to the conditions of stress, reduced water potential in plant cells culture, increased accumulation of nutrients in the cell and finally increased somatic embryogenesis. Proline was found to give optimal responses in the primary and secondary embryogenesis in roses (Marchant *et al*., 1996). Several reports also mentioned the role of proline on somatic embryogenesis in maize (Suprasanna *et al*., 1994) and millet (Vikrant, Rashid, 2002). The present study is the first research that reveals the effect of proline on somatic embryogenesis in *J. curcas*.

Table 4. Effect of proline on secondary somatic embryo proliferation. a, b, c… in the same column show the differences between the means of each of the treatments (Duncan's multiple range test: P<0.05).

Proline (mg/l)	Embryogenesis (%)	Number of embryos/explant	Globular stage	Heart stage	Torpedo stage	Cotyledon stage	Fresh weight (g)
100	80.0b	75.57d	47.53d	18.83d	8.87c	0.33cd	0.084d
200	86.67ab	86.37c	54.1c	21.87bc	9.93bc	0.47bc	0.096c
300	93.33a	111.9a	69.97a	27.53a	13.57a	0.83a	0.125a
400	90.0a	97.53b	61.9b	23.87b	11.43b	0.57b	0.108b
500	86.67ab	81.83cd	50.3d	20.57cd	10.77b	0.17d	0.092c

Table 5. Effect of spermidine on secondary somatic embryo proliferation. a, b, c… in the same column show the differences between the means of each of the treatments (Duncan's multiple range test: P<0.05).

Spermidine (mM)	Embryogenesis (%)	Number of embryos/explant	Globular stage	Heart stage	Torpedo stage	Cotyledon stage	Fresh weight (g)
0.1	90.00ab	102.43c	66.93c	23.83cd	11.17bc	0.50bc	0.113c
0.15	96.67a	129.77a	84.03a	30.13a	14.20a	1.33a	0.144a
0.2	93.33a	115.10b	75.07b	27.03b	12.30b	0.73b	0.127b
0.25	90.00ab	111.03b	74.50b	25.17bc	10.57c	0.47c	0.123b
0.3	83.33b	95.40d	62.60d	22.57d	9.90c	0.33c	0.100d

When spermidine was added to culture medium at concentrations from 0.1 mM to 0.3 mM, the secondary embryos appeared on the surface of primary embryos after five weeks of culture. In all treatments, the concentration of 0.15 mM was suitable for proliferation of somatic embryos via secondary somatic embryogenesis (Table 5). Polyamine is a common compound in cells, and it is involved in the regulation of several processes in plant growth and development, such as stimulation of cell growth, rapidly multiplying cells, creating cloned embryos, root formation, and against stresses (Evans, Malmberg, 1989). Some evidences showed that polyamines were essential for somatic embryogenesis *in vitro* (Mengoli, Bagni, 1992). Spermidine is a special polyamine to be used in somatic embryogenesis in carrot (Feirer *et al*., 1985), *Hevea brasiliensis* (Hadrami, D'Auzac, 1992) and alfalfa (Cvikrová *et al*., 1999). The studies in wild carrot have shown that spermidine plays a direct role in somatic embryogensis and facilitates embryogenesis in culture medium supplemented with compounds which inhibited the synthesis of polyamines (Feirer *et al*., 1985). Research of Kevers *et al*. (2000) also showed that the best somatic embryogenesis in *Panax ginseng* was obtained when 0.1 mM spermidine was used. Paul *et al*. (2009) has studied the embryogenesis in *Momordica charantia*

L. when spermidine was added at very low concentration (0.001 mM). There was no study of effects of spermidine on somatic embryogenesis in *J. curcas* L. In this study, the results showed that the addition of spermidine into culture medium at the concentration of 0.15 mM stimulated somatic embryos formation and proliferation in *J. curcas*.

Plant Regeneration

In the present study, the root formation rates achieved were relatively high in all treatments. On B5 medium, root number, root length and the height of plant achieved were highest (Table 6). MS medium and WPM medium were also suitable for promoting root formation as well as plant growth, but the root length was shorter than that on B5 medium (Fig. 1g, 1h). Many researchers reported the effect of mineral medium components on plant regeneration in different species (Kai *et al*., 2008). MS, B5 and WPM are three of the most common mineral media used for the growth of woody plants. In this experiment, those mineral media gave relatively high root formation rates as well as healthy plantlets. This result is similar to the reports of Moradkhani (2012), Zhang *et al*. (2004) on *Melissa officinalis* L. and *Isatis indigotica* cultured on these media. The comparison of MS, B5 and WPM showed that the phosphorus and calcium levels were similar while the nitrogen content was different. Total nitrogen content of the WPM (14.7 μM) was lower than those in B5 (27.03 μM) and MS (60.03 μM) media (Shi *et al*. 2000). This showed that the total nitrogen content seems like an important factor affecting plant regeneration. Knittel *et al*. (1991) also indicated that additional nitrogen also plays a certain role in shoot regeneration from cotyledon culture in sunflower. In our experiments, the appropriate medium for the formation of root and plantlets development from embryos was B5 mineral medium. This result is consistent with study of Warakagoda and Subasinghe (2009) on shoot regeneration from seeds of *J. curcas*. On B5 medium, mature seeds were germinated and grown better than the immature seeds and fully mature seeds on the MS and WPM medium.

Table 6. Effect of mineral salt formulations on plant regeneration. a, b, c… in the same column show the differences between the means of each of the treatments (Duncan's multiple range test: P<0.05).

Mineral salt formulations	Root formation (%)	Number of roots/explant	Length of root (cm)	Weight of plantlet (g)
1/2MS	80.00a	1.00c	0.54d	0.091c
MS	93.33a	3.33b	1.57b	0.103b
B5	100.00a	4.07a	2.02a	0.125a
White	86.67a	2.87b	1.28c	0.111b
WPM	93.33a	3.07b	1.44bc	0.107b

Table 7. Effect of IBA and NAA on plant regeneration. a, b, c… in the same column show the differences between the means of each of the treatments (Duncan's multiple range test: P<0.05).

PGRs (mg/l)		Number of roots/explant	Length of root (cm)	Weight of plantlet (g)	Height of plant (cm)
NAA	IBA				
-	-	3.47c	1.62g	0.126g	2.31f
0.5	-	3.73bc	1.83fg	0.134fg	2.36f
1.0	-	3.93bc	2.13de	0.142f	2.57de
1.5	-	4.20ab	2.58b	0.251b	3.03b
2.0	-	4.13ab	2.37bcd	0.226c	2.77c
-	0.5	3.67bc	1.99ef	0.103h	2.45ef
-	1.0	4.00abc	2.32cd	0.166e	2.62cd
-	1.5	4.53a	3.02a	0.287a	3.23a
-	2.0	4.00abc	2.45bc	0.215d	2.96b

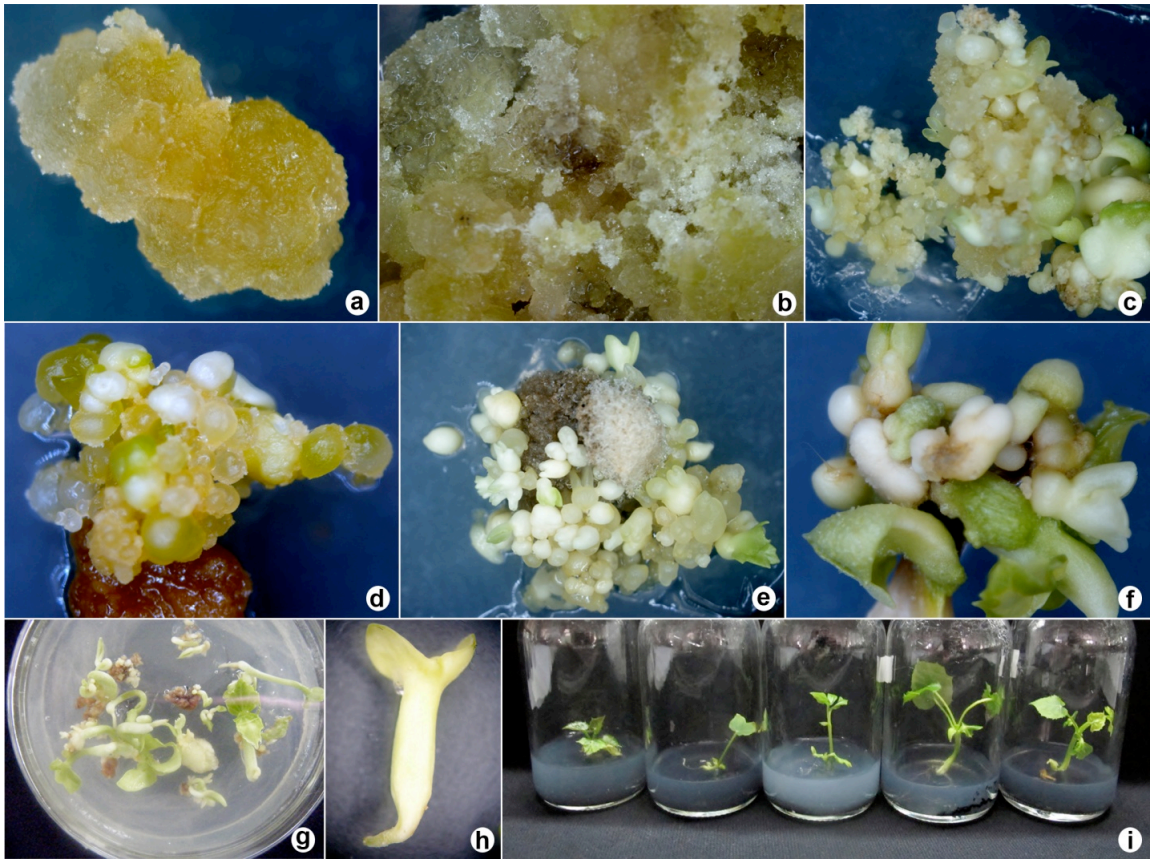

Figure. 1. Primary and secondary somatic embryogenesis of *Jatropha curcas* L.: Embryogenic callus (a, b), Primary somatic embryogenesis from callus (c), Secondary somatic embryogenesis from primary embryos (d), Secondary somatic embryo proliferation (e), Somatic embryo development (f), Plantlet regeneration from secondary embryos (g, h), 4 weeks old somatic embryo derived plantlets (i).

Auxin is an essential of plant growth regulation and stimulates the processing root formation and plant development (Wiesmal *et al*., 1998). The high of endogenous auxin can lead to an increase in root formation rate (Blazkova *et al*., 1997). Auxin also has many effects on the adventitious root formation in some woody plants (De Klerk *et al*., 1999; Diaz-Sala *et al*., 1996). In this study, root formation of *J. curcas* plantlets was observed from the fourth week of culture, and medium supplemented with 1.5 mg/l IBA was shown to be the most suitable medium for plant regeneration (Table 7, Fig. 1i).

The previous studies on plant regeneration in *J. curcas* used culture medium supplemented with adenine sulphate, KIN and IBA for somatic embryogenesis maturation (Jha *et al*., 2007); however, the ratio of mature embryogenesis rate and plant regeneration from secondary somatic embryogenesis obtained were relatively low after six weeks of culture (50% and 20%, respectively). In *J. curcas*, Rajore *et al*. (2007) also reported that medium supplemented with IBA is optimal for stimulating the root formation. In our experiment, medium culture supplemented with 1.5 mg/l IBA resulted in the plant regeneration rate of 100% after four weeks of culture. This result is similar to that of Pandey *et al*. (2011) in *Ginkgo biloba* L. when IBA was added to culture medium to achieve the root formation rate of 100%.

CONCLUSION

This study showed that the somatic embryogenesis was influenced by PGRs, amino acids (proline) and polyamine (spermidine). An efficient somatic embryogenesis system was

established through secondary somatic embryogenesis opening up some new methods for multiplication of embryos and seedlings of *J. curcas*.

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QUÁ TRÌNH PHÁT SINH PHÔI SƠ CẤP VÀ THỨ CẤP CỦA CÂY CỌC RÀO QUA NUÔI CÂY LỚP MỎNG TẾ BÀO CẮT NGANG LÁ

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TÓM TẮT

Hệ thống tái sinh cây Cọc rào thông qua phát sinh phôi sơ cấp và thứ cấp từ mẫu cấy lá *ex vitro* của cây 6 tháng tuổi được trình bày trong nghiên cứu này. Các mẫu lá được cắt lớp mỏng theo chiều ngang (0,5 mm x 10 mm) và được cấy vào môi trường MS có bổ sung KIN (0,5; 1,0; 1,5; 2,0 mg/l) kết hợp với IBA (0,1; 0,5; 1,0 mg/l) hoặc 2,4-D (1,0; 1,5; 2,0 mg/l). Sự hình thành mô sẹo có khả năng sinh phôi đạt tốt nhất trên môi trường có bổ sung 1,0 mg/l KIN và 1,5 mg/l 2,4-D. Các mô sẹo này sẽ được cấy vào môi trường cơ bản MS có bổ sung 2,4-D (0,01; 0,03; 0,05; 0,07 mg/l) hoặc KIN (0,5; 1,0; 1,5; 2,0 mg/l) để tạo phôi sơ cấp. Sự hình thành phôi sơ cấp đạt tốt nhất trên môi trường có bổ sung 1,0 mg/l KIN (76,67%). Các phôi sơ cấp được cấy vào môi trường cơ bản MS có bổ sung KIN (0,1; 0,5; 1,5; 2,0 mg/l) kết hợp với 0,2 mg/l IBA hoặc 0,5 mg/l 2,4-D. Các phôi sơ cấp sẽ tiếp tục hình thành phôi thứ cấp trong môi trường có bổ sung 1,5 mg/l KIN và 0,05 mg/l 2,4-D. Mặc khác, kết quả cũng cho thấy proline (300 mg/l) hoặc spermidine (0,15 mM) có vai trò lên sự gia tăng số lượng phôi thứ cấp khi bổ sung vào môi trường nuôi cấy. Các cây con hoàn chỉnh được tái sinh từ phôi soma khi nuôi cấy trên môi trường B5 (Gamborg, 1968) có bổ sung 1,5 mg.l^{-1} IBA.

Từ khoá: *Cây Cọc rào, proline, spermidine, sự phát sinh phôi soma sơ cấp, sự phát sinh phôi thứ cấp*