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RESEARCH ARTICLE

Efficient Micropropagation Protocol for *Jatropha Curcas* Using Liquid Culture Medium

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Abstract

Although several studies have been made on the micropropagation of *Jatropha curcas* using agar base mediums, none of them have been by using liquid medium systems. The effects of explant type and temporary immersion system (test tube, jar with filter paper boat, and growtek bioreactor) on the micropropagation of *J. curcas* were studied. The explant type influenced shoot quality, multiplication coefficient (MC), and rooting. Leaf explant produced more and longer shoots than nodal explant. Use of filter paper (FB) boat prevented hyperhydricity and allowed proliferation of nodal explants cultured in liquid MS (Murashige and Skoog) medium supplemented 6-benzylaminopurine (BAP) and Kinetin (KN). The best shoot bud induction (92.1±3.1%) was achieved in liquid MS medium supplemented with 2.0 mg/L KN. Leaf regeneration efficiency was compared in growtek bioreactor and in jar containing liquid MS medium supplemented with 0.5 mg/L Thidiazuron (TDZ). The best shoot bud regeneration (78.7±2.1%) was obtained in growtek bioreactor. Shoot buds achieved from nodal segment and leaf were subcultured on filter paper boats in jar and bioreactor containing liquid MS medium supplemented with BAP, Indole butyric acid (IBA), Indole-3-acetic acid (IAA), and KN. Best shoot proliferation and elongation was obtained in filter paper boats containing liquid MS medium supplemented with 1.5 mg/L BAP, 0.5 mg/L IAA, and 0.2 mg/L KN. The number of multiple shoot buds was higher in leaf explants as compared to nodal explants and the highest number of multiple shoot buds was recorded from leaf explants. Up to 76.4% rooting efficiency was obtained when the shoots were *ex vitro* rooted. The generated plants well established in the nursery and grew normally in outdoor conditions. The protocol has good potential for application in large-scale propagation of *J. curcas* using liquid medium.

Key words : Cotton, filter paper boat, bioreactor, liquid medium, jatropha

Introduction

Jatropha curcas is still under the race as second generation biofuels (Navarro-Pineda et al. 2016) *J. curcas* is resistant to drought, and can grow on marginal lands with minimum fertilizer inputs with moderate to high rainfall. Its cultivation will provide employment to rural people and also improve the environment (Openshaw 2000). The plant can be propagated through seeds but for sustainable yield clonal propagations are preferable.

In vitro propagation methods have been used to multiply many plants (Loberant and Altman 2010). However, the use of agar-agar, sucrose, and phytohormones make the *in vitro* propagation system expensive. The cost of micropropagated plant can be reduced by the use of liquid medium, commercial grade sucrose, and *ex vitro* rooting. Also, liquid medium has an advantage over solid medium like faster growth rate, greater number of multiple shoot buds, and no disruption in growth due to phenolic released by explant and contrary solid medium is labor intensives. Many plants respond poorly to liquid medium due to hyper-hydration (Aitken-Christie et al. 1995; Etienne et al. 2006; Shaik et al. 2010; Snyman et al. 2011; Ziv 2010), and solid medium is the only alternate for their propagation. The liquid culture system allows the study of diverse physiological, and biochemical characteristics (Salaj et al. 2007). Recently, the use of suspension culture has also been involved in genetic transformation studies (Wenck et al. 1999). The use of liquid cultures not only

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reduces the cost and labor, but also enhances multiplication rates with or without mechanization (Ascough and Fennel 2004). Cell suspension culture of *J. curcas* has been reported by Soomro and Memon (2007) without any further regeneration from callus. Tissue culture of *J. curcas* have been tried using various explant sources on solid medium by many researchers (Deore et al. 2008; Khemkladngoen et al. 2011; Khurana-Kaul et al. 2010; Kumar et al. 2010, 2011; Kumar and Reddy 2012; Singh et al. 2010, 2014), but the use of liquid medium has remained confined to somatic embryogenesis (Cai et al. 2011; Demissie and Lele 2013). To the best of our knowledge, this is the first report on micropropagation of *J. curcas* using liquid medium. We report here efficient micropropagation method using nodal and leaf explants. High shoot multiplication and shoot growth in liquid medium were optimized using different vessel conditions.

The aim of this study was to develop an efficient micro-propagation protocol using liquid medium. Various temporary immersion systems and explants were tested for best shoot quality, multiplication coefficient and rooting.

Materials and Methods

Plant material and culture condition

Nodal explants were collected from 3-4 year-old elite genotype of *J. curcas* (CSMCRI-5). Nodal explants of 3 cm were excised from young shoots. Explants were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 8 min and rinsed three times in sterile distilled water. Uniform culture conditions were applied to all experiments. The pH of the medium was adjusted to 5.7 prior to autoclaving at 1.05 kg cm^{-2} pressure at 121°C for 20 min. The cultures were maintained at 25 ± 2°C under a 16 h photoperiod with light intensity of 88 µmol m^{-2} s^{-1} (cool white fluorescent tubes).

Shoot bud induction from nodal and leaf explants

Nodal explants were cultured in test tube containing filter paper boat and filled with liquid MS medium supplemented with 1.0-3.0 mg/L Kinetin (KN), and 1.0-3.0 mg/L 6-b*enzyl-aminopurine* (BAP); nodal explants were also cultured in bioreactor (Growtek, Tarson) containing liquid MS medium supplemented with 1.0-3.0 mg/L KN, and 1.0-3.0 mg/L BAP for shoot bud induction. The leaves collected from 2-month-old cultures were used as explants. Leaf explants were cultured in bioreactor, jars containing cotton covered with a filter paper filled with liquid MS medium supplemented with 0.1 -1.0 mg/L Thidiazuron (TDZ) and 1.0-3.0 mg/L BAP for shoot bud regeneration. Percent shoot bud induction and number of shoot buds was recorded after 4 weeks.

Shoot proliferation and elongation

Shoot buds obtained from nodal and leaf explants were cultured in liquid MS medium supplemented with different concentration and combination of 0.5 mg/L BAP, 0.5, 1.5 mg/L Indole acetic acid (IAA), 0.5, 1.5 Indole butyric acid (IBA), and 0.2, 0.5 mg/L KN for shoot proliferation and elongation in bioreactor and jar containing zig-zag (Z) folded filter paper boat. The number of shoots, and shoot length were recorded, after 6 weeks of culture. Multiplication coefficient (MC), calculated as the number of new segments of 1.0 cm (for subculturing) obtained per explant.

Ex vitro rooting and establishment

Elongated shoots (3-4 cm) were harvested and transferred to poly bags containing sterilized sand, wetted with sterile water, and covered with transparent poly bags to maintain humidity. The rooting percentage was recorded, after 4 weeks. Covered poly bags were gradually punctured and finally cut to slowly harden the plants. After 1 week, hardened plants were shifted to the nursery.

The data are provided as mean ± standard error; each treatment comprised 10 explants and repeated three times. Data was analyzed by analysis of variance (ANOVA). Data was statically analyzed using SPSS (7.5). For the figures, standard error (SE) was calculated from the residual variances.

Results

Shoot bud induction from nodal and leaf explants

Best shoot bud response (92.1±3.1%) from nodal explants was achieved on liquid MS medium supplemented with 2.0

Table 1. Effect of different concentrations of PGRs and culture vessel on the percentage of shoot bud induction from nodal explants of *J. curcas*.

BAP (mg/L)	KN (mg/L)	Test tube SBI (%)	Test tube NH (%)	B SBI (%)	B NH (%)
1.0		74.8±3.6c*	89±2.6c	73.5±2.8*b	73±3.5b
2.0		86.7±3.9b*	91±2.1b	78.2±2.6*b	75±3.4b
3.0		84.3±4.6b*	92±2.9b	79.4±3.4*b	78±2.9b
	1.0	76.4±2.6c	100±2.7a	72.4±3.6b	86±2.4a
	2.0	92.1±3.1a	100±1.9a	88.6±3.4a	88±2.1a
	3.0	91.3±3.4a	100±2.4a	87.6±3.5a	86±2.8a

Data were presented as mean ± SE. Means followed by the same letter within columns are not significantly different at the 5% probability level. NH non-hyperhydric, SBI Shoot bud induction, B Bioreactor, BAP 6-benzylaminopurine, KN Kinetin, *callus formation.

Fig. 1. Axillary shoot bud induction from nodal segment in test tube (A), shoot bud regeneration in bioreactor from leaf explants, after 5 days (B-C), shoot proliferation and elongation of shoot buds in jar (D-E), shoot proliferation and elongation of shoot bud in bioreactor (F-G), ex vitro rooted shoots in sterile soil after 4 weeks (H-I).

Table 2. Effect of different concentrations of PGRs and culture vessel on the percentage of shoot bud regeneration from leaf explants of *J. curcas*.

BAP (mg/L)	TDZ (mg/L)	B SBI (%)	B NH (%)	Jar SBI (%)	Jar NH (%)
1.0		36.4±3.3d	78.2±2.3b	21.1±3.4c	67±1.6b
2.0		56.8±3.5c	76.1±1.9b	33.6±3.6b	66±2.6b
3.0		41.3±3.4d	77.6±2.6b	31.2±3.1b	68±2.9a
	0.1	66.4±2.3b	96.2±2.1a	32.3±2.8b	69±3.1a
	0.5	78.7±2.1a	99.1±3.2a	46.2±2.6a	71±2.8a
	1.0	75.1±2.6a	97.3±2.8a	41.1±2.4a	73±1.8a

Data were presented as mean ± SE. Means followed by the same letter within columns are not significantly different at the 5% probability level. SBI Shoot bud induction, B Bioreactor, NH non-hyperhydric, BAP 6-benzylaminopurine, TDZ Thidiazuron.

Table 3. Effect of hormones and culture system on growth response and rooting of leaf cultures.

IAA (mg/L)	BAP (mg/L)	KN (mg/L)	B MC	Jar MC	B SL (cm)	Jar SL (cm)	B RS	Jar RS
0.5	0.5	-	1.1±0.1b	2.6±0.2c	1.1±0.5a*	1.5±0.4b*	0.8±0.1a	1.3±0.2b
1.5	0.5	-	1.7±0.3a	2.4±0.4c	1.3±0.4a*	1.9±0.6b*	0.6±0.2b	1.5±0.3b
1.5	0.5	0.2	2.3±0.2a	8.6±0.2a	1.8±0.6a	3.4±0.4a	1.3±0.3a	2.4±0.1a
1.5	0.5	0.5	2.1±0.4a	3.9±0.6b	1.4±0.5a	2.1±0.5b	0.9±0.1a	2.1±0.5a

Data were presented as mean ± SE. Means followed by the same letter within columns are not significantly different at the 5% probability level. *callus formation, MC multiplication coefficient, SL length of the longest shoot, RS number of rootable shoots per explant, B Bioreactor, BAP 6-benzylaminopurine; IAA Indole-3-acetic acid; KN Kinetin.

mg/L KN (Table 1). BAP developed callus at the proximal end of the explants in all the treatments. However, no callusing was observed in all the treatments of KN containing medium (Fig. 1A). Shoot buds initiated in bioreactor but did not elongate further. This may be due to the orientation of the nodal explants. Nodal explants were lying horizontally in bioreactor due to broader surface area. The percentage of non-hyperhydric (NH) shoot was more in test tube as compared to bioreactor (Table 1). Hence, test tubes were more suitable for shoot bud induction as compared to bioreactor.

Best shoot bud regeneration from leaf explants was achieved on liquid MS medium supplemented with 0.5 mg/L TDZ in bioreactor. Poor shoot bud regeneration was noted in leaf cultured on liquid MS medium supplemented with 0.5 mg/L TDZ in jar. Shoot bud initiation was started on the 5th day in bioreactor (Figs. 1B, C). Optimum shoot bud regeneration (78.7±2.1%) was noted in bioreactor as compared to 46.2± 2.6% in jar (Table 2). The percentage of non-hyperhydric (NH) shoots was more in bioreactor (99.1±3.2) as compared to jar (71±2.8). Role of culture vessel was critical for optimum shoot bud regeneration of leaf explant. BAP resulted poor shoot bud regeneration in all the treatments.

Shoot proliferation and elongation

Shoot buds were cultured in bioreactor and jar containing liquid MS medium supplemented with BAP, IBA, IAA and KN. The combination of 0.5 mg/L BAP and 1.5 mg/L IAA developed callus and addition of 0.2 mg/L KN to the same medium minimized the callus (Table 3). Optimum shoot proliferation and elongation was achieved in jar (Figs. 1D, E) as compared to bioreactor (Figs. 1F-G) from leaf explant. Greater shoot length (3.4 cm) in jar was noted over bioreactor (1.8 cm) using leaf explant, filter paper folding might have provided better support and enhanced shoot growth. Shoot proliferation and elongation response of axillary shoot buds was poorer compared to leaf regenerated shoot buds in both the culture vessel. Highest multiplication coefficients of 8.6±0.2 and number of rootable shoots per culture 2.4±0.1 were recorded from leaf explants in jar (Table 3). Poor multiplication coefficients of 2.3±0.4, and number of rootable shoots per culture 1.7±0.1 were recorded from nodal cultures in jar (Table 4). Leaf cultures multiplied more rapidly than nodal cultures. Also, shoot length of leaf cultures was better than nodal cultures. These results revealed that leaf is better explant for micropropagation of *J. curcas* using liquid medium. It was also known by the results that shoot proliferation and elongation was affected by different culture system and explant type. Shoots were further subcultured on the same liquid medium to get sufficient shoot length for rooting.

Ex vitro rooting and establishment

Shoots were harvested from both the explant types leaf and nodal, growing in liquid medium containing 0.5 mg/L BAP, 1.5 mg/L IAA, and 0.2 mg/L KN. Poor rooting of 36.1% (leaf cultures) and 34.1% (nodal cultures) was noted in shoots obtained from the bioreactor (Fig. 2). This may be due to hyperhydric malformations in bioreactor. Best rooting (76.4%) was achieved from shoots of leaf cultures obtained from jar. Plantlets resume shoot growth and there were new leaves on each plant, after transferring in poly bag. The plantlets exhibited good shoot development and root growth (Figs. 1H, I), which confirmed the ability of shoots derived from liquid medium to root and to continue growth, after transplant to soil. The acclimatized plantlets were well established upon transfer to greenhouse. More than 90% plant survival was obtained in the greenhouse and nursery.

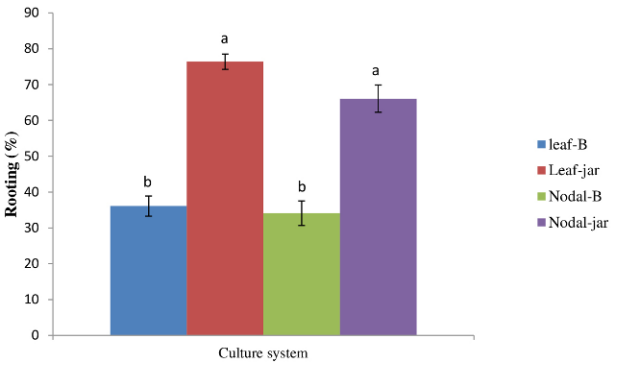

Fig. 2. Effect of culture system on ex vitro rooting of *Jatropha curcas*.

Table 4. Effect of hormones and culture system on growth response of nodal cultures.

BAP (mg/L)	KN (mg/L)	IAA (mg/L)	IBA (mg/L)	B MC	Jar MC	B SL (cm)	Jar SL (cm)	B RS	Jar RS
0.5	-	0.5	-	0.5±0.1b	1.1±0.4b	0.6±0.3b	1.1±0.4b	0.5±0.1a	0.6±0.2b
0.5	-	1.5	-	0.7±0.2a	1.4±0.3b	0.8±0.4a*	1.3±0.5b*	0.6±0.2a	0.7±0.1b
0.5	0.2	1.5	-	1.2±0.1a	2.3±0.4a	1.5±0.4a	2.3±0.6a	1.1±0.1a	1.7±0.3a
0.5	0.5	1.5	-	1.4±0.2a	1.8±0.2a	1.1±0.6a	1.8±0.4a	0.9±0.1a	1.6±0.1a
0.5	0.2	-	0.5	1.3±0.3a	1.1±0.3b	0.5±0.4b*	0.8±0.b3*	0.6±0.3a	0.8±0.1b
0.5	0.5	-	1.5	1.1±0.4a	1.5±0.1b	0.6±0.3b*	0.9±0.5b*	0.7±0.1a	0.8±0.3b

Data were presented as mean ± SE. Means followed by the same letter within columns are not significantly different at the 5% probability level. *callus formation, MC multiplication coefficient, SL length of the longest shoot, RS number of rootable shoots per culture, B Bioreactor. BAP 6-benzylaminopurine; IAA Indole-3-acetic acid; IBA 3-indolebutyric acid; KN Kinetin.

Discussion

Plant production using liquid medium is a harmonized system to overcome limitations present in the agar-based solid medium (Aitken-Christie 1991; Paek et al. 2001, 2005). To date, there was no study reported on the micropropagation of the *J. curcas* in liquid cultures using nodal and leaf explants. Thus, in the current study, we investigated the possibility of micropropagation in liquid medium and assessing different immersion methods together with various PGR combinations. In solid medium, shoots absorb nutrients through their cut end (Guan and De Klerk 2000), however, the medium taken up by plants in a liquid medium is through leaves via stomata and transferred to growing tissues (De Klerk and Ter Brugge 2011; Schonherr 2006). Liquid medium uptake over the whole plant surface can be beneficial for the growth of plantlets (Berthouly and Etienne 2005; Quiala et al. 2006). In our study, vessel type significantly affected initiation and elongation of shoot buds. The medium volume and the culture vessel type were critical for the micropropagation of sugarcane (Snyman et al. 2011). We observed highest leaf regeneration in bioreactor as compared to jar. Similar to the above observations, improved plant regeneration in liquid media using different culture vessels were earlier noted in several plants (Mujib et al. 2014; Nitayadatpat and Te-chato 2005; Te-chato and Lim 1999). This fact could explain our results, that liquid medium improved shoot proliferation and elongation as best shoot proliferation was achieved in jar containing liquid medium. Sometimes the permanent contact of plant tissues with a liquid medium causes total or partial hyperhydricity (Etienne et al. 2006; Niemenak et al. 2008). In the present study, such effects could be overcome by the use of test tube and filter paper boat. Test tube and boat provided better support to shoots to maintain upright position as compared to bioreactor. The only lower end of the nodal explant was in the contact with medium; hence, more number of non-hyperhyderated shoots was achieved from nodal explants in test tube. Liquid medium improved regeneration efficiency of *Centaurium erythraea* and *Phaseolus vulgaris* (Piatczak et al. 2005; Veltcheva and Svetleva 2005). In the present study, BAP-containing medium induced callusing at the basal end of the explants. However, no callusing was noted in the explants cultured on KN-containing medium. Cytokinins in their higher concentrations have increased the occurrence of vitrification (Ivanova and Van Staden 2011). Vitrified shoots and leaves were observed in BA containing medium in teak (Quiala et al. 2012). BAP along with IAA developed intervening callus and addition of KN to the same medium inhibited callusing. Similar observation was noted in sugarcane liquid cultures, BAP in combination with KN boosted up culture growth (Singh et al. 2001). Advantage of the use of liquid medium was to drop down the costs in media constituents, media preparation, and waste disposal, together with less manual labor for inoculation of cultures.

To date, there was no study reported on the micropropagation of the *J. curcas* in liquid cultures medium. It was known by the study that the use of a support to maintain the explants in an upright position was essential to minimize hyperhydricity, and it was the main obstacle for propagation in liquid medium. Three types of temporary immersion system were evaluated and all of them proved suitable at different culture stages. The test tube was suitable for nodal explants, bioreactor was best for leaf regeneration and jars produced longer shoots. Thus, in the current study, we investigated the possibility of micropropagation in liquid medium by assessing different explants, immersion types and together with various PGR combinations.

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