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IMMATURE EMBRYO IS THE POTENTIAL SOURCE FOR *IN VITRO* PLANT REGENERATION IN *JATROPHA CURCAS*

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Abstract

Context: *Jatropha* belongs the spurge family Euphorbiaceae. Special interest mounting for its biodiesel which has created enthusiasm in cultivation of the species for oil extraction.

Objectives: The study was conducted to develop the protocol for tissue and callus culture in Bangladeshi *Jatropha curcus* plant particularly to identify the most suitable explants for its wide scale micropropagation.

Materials and Methods: Immature embryos taken from four developmental stages of fruits were cultured on growth regulator free MS liquid medium. After fifteen days of germination, elongated hypocotyls and two cotyledonary leaves were used as explants.

Results: Embryo derived seedlings acted as the potential source of explants both for callus and plantlets. The immature embryo of size 0.87cm produced highest callus formation (83.33%) on MS medium supplemented with lower concentration of 2, 4-D (0.5 mg/l) and coconut water 2% (v/v). Immature embryos grown on MS basal medium supplemented with 2,4-D (0.2 mg/l, 0.5 mg/l and 1.0 mg/l) alone or in combination with coconut water 2% (v/v) exhibited a wide range of callus induction percentage (26-100%) for hypocotyls and (20 - 40%) for cotyledonary leaves.

Conclusion: The age of immature embryo and addition of growth adjuvants and growth additive to the culture medium played the role in promoting better callus and plantlet formation.

Keywords: *Jatropha curcus*, immature embryos, *in vitro* culture, biodiesel.

Introduction

Jatropha curcus L., widely grown in Bangladesh, is commonly known as physic nut, jamalgota, ratanjot or purgative nut. It is widely distributed in tropical and subtropical areas (Schmook and Serralta-Peraza 1997). This multipurpose tree has received global attention due to its seeds which contain 40-50% semi drying oil which is recently used as efficient substitute for diesel fuel (Takeda 1982, Banerji *et al.*1985, Martin and Mayeux 1982, Muhlbauer *et al.* 1998). But this oil is not edible due to the presence of toxic substance "Curcuscine" (Gandhi *et al.*1995) and is named biodiesel that can be used as a direct replacement for kerosene for cooking and lighting, as an engine fuel (Hussain *et al.* 2009). Realizing the importance of renewable energy sources country like Cape Verde, Madagascar, Nicaragua, Brazil, Mali are using the seeds of *J. curcus* for large scale production of biodiesel (Heller 1996).

Poor seed germination due to toxic chemicals in the seeds and seed protein, latex, endophytic bacterial contamination raise serious problems for its micropropagation by tissue culture. Therefore efforts for the last two decades have failed to provide a reliable protocol of *in vitro* plantlet formation of *J curcus* (Jha *et al.* 2007, Deare and Johnson 2008). Again, despite the research efforts over the past few years in *J. curcus* tissue culture, no facile well developed protocol of regeneration has been developed so far (Sujatha and

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Mukta 1996, Sujatha *et al.* 2005, Rajore and Batra 2007, Jha *et al.* 2007,Deare and Johnson 2008). In most of the cases workers used shoot tip, nodal segment and hypocotyl as explants in *in vitro* regeneration of *J. curcus*. Here we have developed an elaborate and standard method for proliferation of immature embryos towards regeneration of plantlets as well as its callus culture.

Materials and Methods

Plant materials

A field survey was made throughout Bangladesh to collect the available germplasms of *Jatropha* and grown in the research campus of the Institute of Biological Sciences, University of Rajshahi, Bangladesh. Fruits were collected from *Jatropha* research field of the Institute of Biological Sciences, University of Rajshahi, Bangladesh. Fruits were identified on 4, 5, 6 and 7 weeks after pollination (WAP) and were accordingly classified as four groups. The fruit colour, fruit size, seed size and embryo size were the criteria used to select the right stage of the explants. First two classes were characterized by green fruit colour but third and fourth classes were characterized by dark green and yellowish fruit colour respectively (Table 1).

Methods

Immature embryo isolation

Seeds were removed from fruits with a surgical scalpel and were cleaned with tap water and then soaked in distilled water for 12 hrs. The soaked seeds were washed with distilled water for 20 minutes. The seeds were then gently cracked to expose the zygotic embryos surrounded by a kernel and kept in distilled water for 6 hrs to run out the oily substances from the kernel. After washing five times the decoated seeds were immersed in 70% ethanol (v/v) for 30 seconds followed by successive washing with sterile distilled water 3 times in a laminar flow. The decoated seeds were then treated with 0.1% mercuric chloride (w/v) for 15 minutes and finally rinsed five times with sterile water. The immature embryos (embryonal axis + cotyledons) were aseptically removed from the seeds and transferred to the paper bridge (Fig.1).

Embryo germination

The embryos were germinated on growth regulator free MS (Murashige & Skoog 1962) liquid medium supported with filter paper bridge (M shaped) in test tube. All media had 3% sucrose (w/v) supplemented with different concentrations of coconut water (CW) and pH was adjusted prior to autoclaving. The test tubes were incubated in dark at 28°C for 2 days and when hypocotyls was elongated the test tubes were transferred to light intensity of 2000 lux provided by cool and florescent lamps. In the light regime the embryos were germinated with an elongated hypocotyl having two cotyledonary leaves at the top (Fig.1) within 15 days. Finally the germinated seedlings were used as the potential source of explants.

Callus induction

Fifteen-day-old seedlings (including cotyledonary leaves and hypocotyls) were excised aseptically and cultured on solid MS medium supplemented with growth regulators and coconut water (v/v) for induction of callus. The media also contained 3% sucrose (w/v) supplemented with a number of adjuvants like casein hydrolysate (CH) 100 mg/l, L-glutamine 200 mg/l, cupper sulphate 8 mg/l. Cultured media were maintained in growth room at 50-60% relative humidity and 25°C ±2°C temperature under controlled light intensity of 2000 lux. Initially after one week of culture green soft friable callus grew at the margin of hypocotyls. The callus was then maintained under the same conditions with subcultures at 3 weeks interval in fresh medium. On the

other hand cotyledonary leaves also showed callus initiation after two weeks of culture that was also maintained with subcultures at 3 weeks intervals. Percentage of callus formation, texture, colour and their respective growth from hypocotyls and cotyledonary leaves were recorded and presented in Table 3.

Plantlet formation

Growing seedlings developed from immature embryo were also used for plantlet formation. MS medium supplemented with different concentrations of BAP along with growth additive like polyvinyl pyrrolidone (0.5g/l). Cultured media were kept in growth chamber 25°C ± 2°C and 50-60% relative humidity under controlled light intensity of 2000 lux by a light assembly consisting of 18/6 hrs L/D cycle. After seven days shoot length, leaf initiation time and plantlet height were recorded (Table 5). A schematic representation of callus induction and plantlet formation from immature embryos are shown in Fig.below:

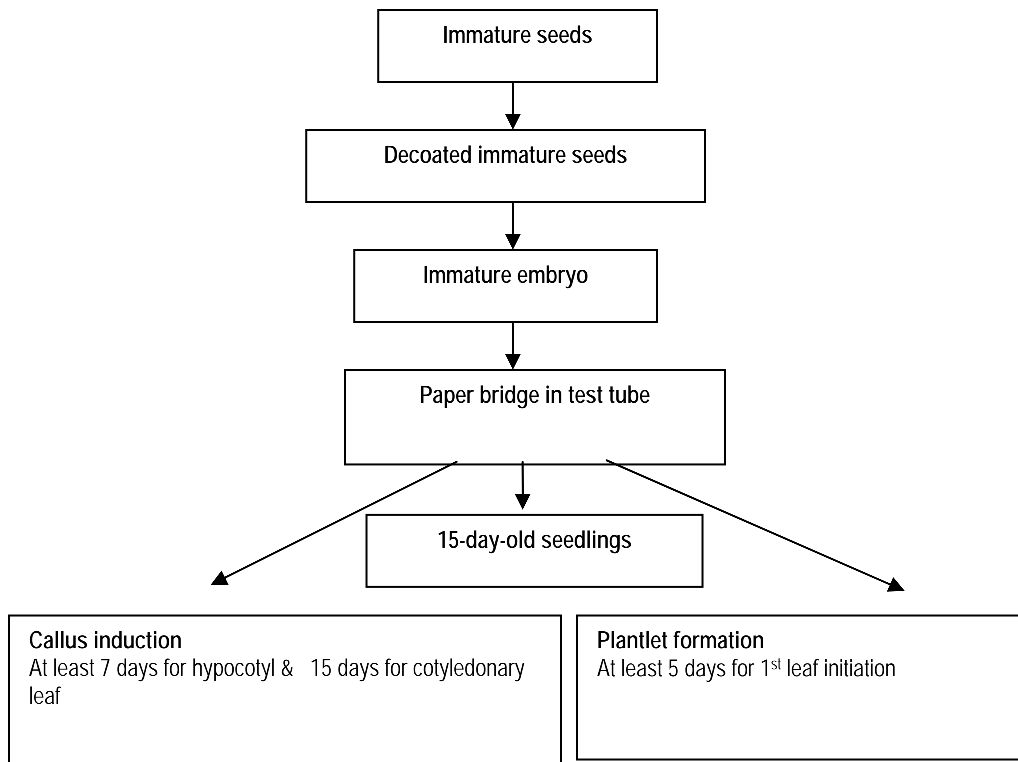

Fig.1. Schematic representation of optimized protocol for callus induction and plantlet formation from immature embryo in *J. curcas.*

Results and Discussion

Immature embryo size and their germination

Four size classes of immature embryos (0.50 cm, 0.82 cm, 0.87 cm and 0.97cm in length) were cultured on growth regulator free MS liquid medium supplemented by filter paper bridge (M- shaped) in test tube. There were significant differences in germination among the different classes. The immature embryo size class-3 (0.87 cm) obtained from dark green coloured fruits of six WAP showed highest germination (50%) on paper bridge (Fig.1).

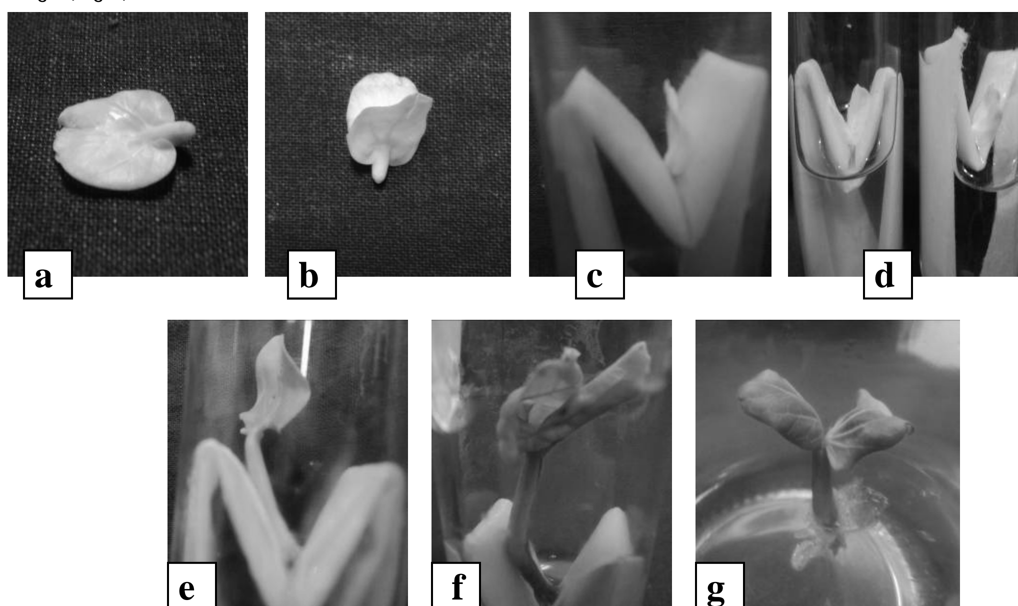

Fig. 1. Germination of embryo on M-shaped filter paper bridge with growth regulator free MS liquid medium. **a.** Complete embryo; **b.** Embryo with exposed cotyledons; **c-d**. Embryo on M-shaped filter bridge; **e.** Germinated embryo; **f.** Embryo with two cotyledonary leaves and **g.** 15-day-old seedling.

Table 1. Effect of embryo size and their germination under different fruit growths

Age of the fruits (WAP)	Colour of fruits	Size class of the fruits	Size of fruits (cm)	Size of seeds (cm)	Size of embryo (cm)	Germination (%)	Survival (%) after germination
4	Green	1	2.5	1.64	0.5	0	0
5	Green	2	2.8	1.75	0.82	0	0
6	Dark green	3	3.02	1.82	0.87	50.0	80.0
7	Yellowish	4	3.22	1.87	0.97	33.33	69.23

WAP- Weeks After Pollination

Germination percentage under *in vitro* condition of *J. curcus* embryo was hard to achieve, since almost first two classes of embryos failed to germinate. Third and fourth classes with embryo length 0.87 cm and 0.97 cm obtained from dark green and yellowish colour fruits respectively achieved 50.0% and 33.33% germination. But other two classes failed to germinate. The results clearly indicated that size of embryo is important in germination. Dark green fruit color after six WAP with embryo size 0.87 cm proved more suitable for germination and also for their subsequent survival. After six WAP germination percentage of the embryo decreases with the increase of embryo size. Embryo size 0.5 cm and 0.82 cm obtained from green fruits of 4 to 5 WAP failed to germinate.

Effect of age of immature embryo on callus induction and plantlet formation

To determine the effect of the age of the immature embryo on germination, four size classes of fruits were taken after 4, 5, 6 and 7 weeks of pollination (WAP). 15-day-old seedlings derived from the immature embryo (0.87cm and 0.97cm) of 6 and 7 WAP of *J. curcus* were cultured on MS basal medium supplemented with different concentrations of 2, 4-D alone and in combination with coconut water. There were significant differences in callus formation among the different ages of embryos. The immature embryo obtained from 6 WAP fruits produced highest callus formation (83.33%) on MS medium supplemented with lower concentration of 2, 4-D (0.5 mg/l) and coconut water 2% (v/v). Again the subsequent plantlet regeneration was also highest (75.0%) in this class of embryo in the same medium (Table 2).

Table 2. Effect of age of immature embryo on callus and plantlet formation in *J. curcus.*

Age of fruit (WAP)	Size class of the fruits	Callus formation (%)	Plantlet formation(%)
4	1	0	0
5	2	0	0
6	3	83.33	66.66
7	4	71.42	75.0

WAP-Weeks After Pollination

Effect of growth regulators on callus induction

Different concentrations of growth regulator with coconut water 2% (v/v) were tested to define an efficient medium for callus induction from immature embryo culture of *J. curcus.* Initially hypocotyls and leaf explants of 15-day-old seedlings of *J. curcus* were cultured on MS basal medium supplemented with different concentrations of 2, 4-D (0.2 mg/l, 0.5 mg/l and 1.0 mg/l) alone and with coconut water 2% (v/v).Callus initiation took place from the cut edges of each hypocotyls explants at 7 days and from leaf explants at 15 days (Fig. 2).

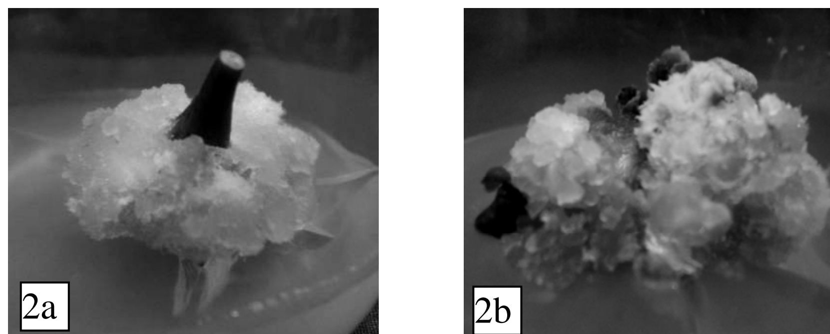

Fig.2. Initiation of callus from the cut edges of hypocotyl and cotyledonary leaf explants in *J. curcus* seedling grown *in vitro* with 0.5 mg/l 2, 4-D and coconut water 2% (v/v). **2a.** Callus initiation from hypocotyl after 7 days and **2b**. Callus initiation from cotyledonary leaves after 15 days of culture.

Callus produced from hypocotyls explants grew faster during first 7 to 30 days of culture and later stabilized at a slow growth rate up to the rest 7 weeks. Similar result was also found by Monacelli *et al.* (1995) in *J. curcus*. On the other hand cotyledonary leaves showed faster callus formation during 15 to 35 days of culture and continued at a slow motion up to the rest 7 weeks medium with 0.5 mg/l 2, 4-D alone showed 40% callus formation from cotyledonary MS leaf explants and hypocotyls showed 100% callus formation (Table 3).

Table 3. Growth regulator formulation used for the induction of callus from 15-day-old seedlings in *J. curcus.*

Growth regulators concentration (mg/l)	15-day-old seedlings					
	Hypocotyls			Cotyledonary leaves		
	Days to callus initiation	% of callus at 7 weeks	Callus response	Days to callus initiation	% of callus at 7 weeks	Callus response
2,4-D 0.2	15	26	White yellow, hard, compact	29	34	Light green, compact, hard
2,4-D 0.2 Coconut water 2%(v/v)	11	48	White yellow, soft, friable, globular	23	20	Light to dark green, soft, nodular
2,4-D 0.5	14	90	Light green, compact, soft, friable	21	40	Light to dark, soft, compact, globular
2,4-D 0.5 Coconut water 2% (v/v)	7	100	Light green, compact, nodular, soft, friable	15	35	Dark green, nodular, compact, hard
2,4-D 1.0	12	65	Light green, compact, slightly soft, globular	24	25	Light to dark green, compact, nodular
2,4-D 1.0 Coconut water 2%(v/v)	9	70	Light green, compact, slightly hard, globular	21	30	Light green, nodular, slightly soft

Callus obtained from immature embryo were easily proliferated in culture (Fig.3). Callus produced from cotyledonary leaves were light to dark in colour, soft compact and globular in shape and callus obtained from hypocotyls showed light green, compact nodular, soft and friable that were similar to the observation of Rao *et al.* (2006).

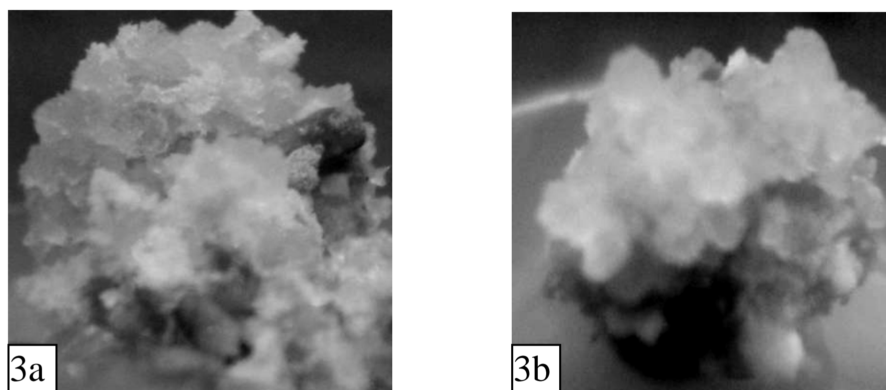

Fig.3. Proliferated callus from hypocotyl and cotyledonary leaves of *J. curcus*. **3a**. Callus from hypocotyl and **3b.** Callus from cotyledonary leaves.

Callus culture was maintained by subculturing after every 3 weeks, otherwise the callus became dark and growth ceased. The darkening of callus was probably due to the production of phenolic substances released by explants. Many workers have emphasized that phenolic substances are responsible for darkening of callus (Monacilli *et al.*1995). The experimental results confirmed that MS medium supplemented with 0.5 mg/l 2, 4-D with coconut water 2% (v/v) proved to be more effective for establishment of callus on a large scale in short period of time. Under different concentrations of coconut water 2% (v/v) was also proved effective in callus induction (Table 4**).**

Table 4. Effect of coconut water on the growth of germinated embryo of *J. curcus*

Media	Days after setting onto the paper bridge in the test tubes			
	5 DAS	8 DAS	11 DAS	14 DAS
MS	0.92	1.11	1.58	1.77
MS + CW (1%)	0.97	1.22	1.71	1.84
MS + CW (2%)	1.16	1.51	1.92	2.1
MS + CW (3%)	1.09	1.37	1.82	1.98

DAS - Days After Setting
CW - Coconut Water

Kawak *et al.* (1995) and Hoshino *et al.* (1995) used coconut water in callus induction in some plants.The other concentrations of 2, 4-D (excluding 0.5mg/l 2, 4-D) used alone and with coconut water 2% (v/v) showed very slow response in callus formation in hypocotyls and as well as in cotyledonary leaves within 7 weeks.

Effect of growth regulators on plantlet formation

15-day-old seedlings of about 2.0 cm height with 2 leaves were cultured on MS medium supplemented with BAP (6-15 mg/l) for 5 weeks. The results on the effect of different concentrations showed that shoot length at 35 DAS gave the highest mean value by applying 12 mg/l BAP followed by 28 DAS, 21 DAS, 14 DAS and 7 DAS respectively (Table 5).

Table 5. Effect of different concentrations of BAP on elongation of proliferated shoots, initiation of leaf and height of plantlet in *J. curcus.*

BAP Conc.	Shoot length (cm)					Time taken for leaf initiation (days)				Plantlet height (cm)				
	7 DAS	14 DAS	21 DAS	28 DAS	35 DAS	1st leaf	2nd leaf	3rd leaf	4th leaf	7 DAS	14 DAS	21 DAS	28 DAS	35 DAS
6 mg/l	2.13	2.22	2.40	2.70	2.89	9-13	16-18	20-23	24-28	2.38	2.63	2.89	2.95	3.01
9 mg/l	2.16	2.30	2.52	2.74	2.99	7-11	15-17	18-20	21-25	2.44	2.78	3.13	3.27	3.42
12 mg/l	2.23	2.47	2.73	2.94	3.11	5-9	12-14	15-17	20-25	2.54	2.99	3.44	3.67	3.91
15 mg/l	2.19	2.15	2.67	2.83	3.05	8-12	14-16	17-19	22-27	2.50	2.85	3.32	3.54	3.65

The results showed that 1st, 2nd, 3rd and 4th leaf initiation time decreased with an increase in concentration of BAP. Finally 12 mg/l BAP concentration took the lowest initiation time for 4th leaf whereas 15 mg/l BAP concentration showed maximum time for leaf initiation. At 12 mg/l BAP concentration leaves were enlarged and finally 3rd and 4th leaves were originated within 15-17 days and 20-25 days respectively. MS medium having 12 mg/l BAP exhibited 2.54 cm of plant height at 7 DAS and finally at 35 DAS it was 3.91 cm in height. However, the shoot length varied with BAP concentration and 12 mg/l BAP was best suited for maximum shoot length. According to Thepsamiran *et al.* (2007) higher concentration of BAP combined with IBA provided less number of shoots and leaves in *J. curcus*. But our study showed that BAP at higher concentration gave the lowest shoot length and maximum time for leaf initiation.

Plantlet height showed that mean values were also highest in 15mg/l BAP followed by 9 mg/lBAP and 6 mg/lBAP respectively. Plant height was maximum (3.91cm) at 35 DAS followed by 28 DAS, 21 DAS, 14 DAS and 7 DAS respectively in 12 mg/l BAP.

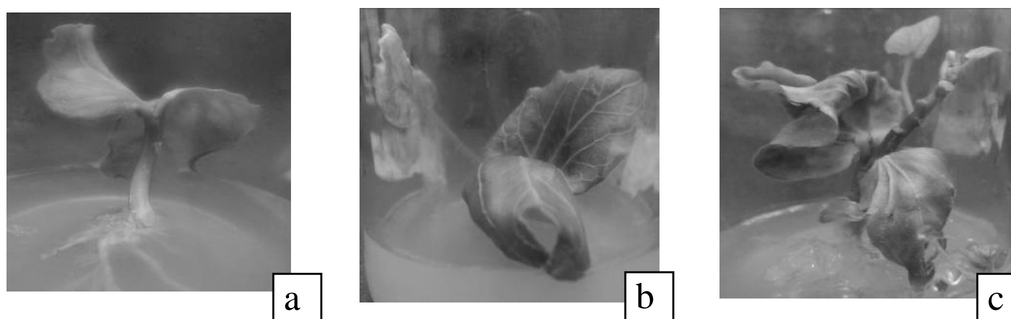

Plate 2. Successive stages of complete plantlet formation from 15-day-old seedling on MS medium supplemented with 12mg/l BAP after 5 weeks. **a**. Seedling with 2 cotyledonary leaves and roots; **b.** Seedling with 3 leaves with roots and **c.** Complete plantlet.

Optimization of callus induction medium and influence of growth adjuvants

Influence of growth adjuvants with different concentrations of 2, 4-D were observed to identify the suitable protocol for callus induction with its maximum extent. For that purpose different concentration of 2, 4-D were used alone or in combination with coconut water 2% (v/v) in supplementation to MS basal medium for callus induction. Two types of callus induction were observed. Some of the calli were green in colour and compact which was morphogenic callus, but in other cases, friable and cream callus was formed, which was non morphogenic callus. Media formulation with 0.5 mg/l 2,4-D and coconut water 2% (v/v) proved most appropriate for induction of more morphogenic callus from hypocotyls and 0.5mg/l 2,4-D alone was rather more suitable for better morphogenic callus induction in case of cotyledonary leaves and these mediua were termed as callus induction medium for hypocotyls (CIM-1) and cotyledonary leaves (CIM-2) respectively. Subsequently further experiments were carried out with 2, 4-D at different doses in combination with coconut water (Table 4). Immature embryos grown on MS basal medium supplemented with 2,4-D (0.2 mg/l, 0.5 mg/l and 1.0 mg/l) alone or in combination with coconut water 2% (v/v) exhibited a wide range of callus induction percentage (26-100%) for hypocotyls and (20 - 40%) for cotyledonary leaves.

For upgrading the medium, CIM-1 was supplemented with growth adjuvants like L-glutamine, casein hydrolysate, copper sulphate alone or in combination with coconut water 2% (v/v). Cultures grown on the media with these additives showed elevated range of callus formation which was increased by 26-100% for hypocotyls and 20-40% for cotyledonary leaf explants. However the maximum morphogenic calli were found in CIM-1 and CIM-2 media supplemented with casein 100 mg/l hydrolysate, 200 mg/l L- glutamine and 8.0 mg/l copper sulphate. Hence the protocol developed in the present study may have a great role in enhancing callus induction as well as plantlet formation in *J. curcus* using immature embryo as the potential source of explants.

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References

Deare AC, Johnson IS. 2008. High frequency plant regeneration from the leaf disc cultures of *Jatropha curcus* L.: an important bio diesel plant. *Plant Biotech Rep* 2, 10-15.

Ghandi A, Chaudhory DR, Reddy MP, Rao SN, Chikara J, Pandya JB.1995. Prospects *Jatropha* methyl ester (bio diesel) in India. *Int J Environ Stud* 64, 659-674.

Heller J. 1996. Physic nut. *Jatropha curcus* L. Promoting the conservation and use of underutilized and neglected crops. I. Institute of Plant Genetic and Crop Plant Research, Gatersleben, International Plant Genetic Resource Institute, Rome.

Hosshino, Yoichioro, Masaru, Nakano, Masahiro M. 1995. Plant regeneration from cell suspension derived protoplast of *Saintpaulia ionanthya* Wendi. *Plant Cell Report* 14(6), 341-344.

Hussain MD, Khan F, Haque MA, Islam MP. 2009. Physical, mechanical properties and oil content of selected indidenous seeds available for Biodiesel production in Bangladesh Agricultural Engineering International: *the CIGR E journal*. XI: 1419.

Jha T, Mukherjee P, Datta MM. 2007. Somatic embryogenesis in *Jatropha curcus* Linn., an important bio-fuel plant. *Plant Biotech Rep* 1, 135-140. http://dx.doi.org/10.1007/s11816-007-0027-2

Kawk Sang S, Soo KK, Moon SL, Kyung HJ. 1995. Acidic per oxidases from suspension cultures of sweet potato. *Phyto-Chemistry (Oxford)*. 39(1), 981-984.

Monacelli B, Pasqua A, Cuteri A, Varusio B, Batta Monache GD. 1995. Histological study of callus formation and optimization of cell growth in *Taxus Baccata*. 159 *Cytobios* 81, 159-170.

Muhlbauer W, Esper A, Stumpf E, Baumann R. 1998. Workshop Report -Rural energy, quity and employment: Role of *Jatropha curcus.* The Rook feller Foundation Scientific Centre (SIRDC), Zimbabwe, May, 13-15.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassys with tobacco tissue cultures. *Physiologia Plantarum* 15, 473-479. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x

Rajore S, Batra A. 2007. An alternative source for regenerable organogenic callus induction in *Jatropha curcus*. *Ind J Biotech* 6, 545-548.

Rao AQ, Hussnain S, Sartraz, Shahzad M, Sadquib, Bakhari S, Yassir A, Hassain T. Rizuddin. 2006. Somatic embryogenesis in wild relatives of cotton (*Gossypium spp*). Journal of Zhejiang University Science. 290-298 pp.

Schmook B, Serralta-Peraza L. 1997. *J. curcus*: distribution and uses in the Yucatan Peninsula of Mexico. In: Gubitz GM, Mittelbach M. Trabi M (eds). Biofuels and industrial production from *Jatropha curcus* Dbv. Verlag Graz 53-57 pp.

Sujatha M, Mukta N. 1996. Morphogenesis and plant regeneration from tissue cultures of *Jatropha curcus. Plant Cell Tissue Organ Cult* 44,135-141. http://dx.doi.org/10.1007/BF00048191

Sujatha M, Makkar HPS, Becker K. 2005. Shoot bud proliferation from axillary nodes and leaf sections of non toxic *Jatropha curcus* L. *Plant Growth Reg* 47, 83-90. http://dx.doi.org/10.1007/s10725-005-0859-0

Takeda Y. 1982. Development study on *Jatropha curcus* (Sabudum) oil as a substitute for diesel engine oil in Thailand. *J Agric Assoc China*. 120, 1-8.

Thepsamiran N, Thepsithar C, Thangpukdee A. 2007. *In vitro* multiple shoot induction of physic nut (*Jatropha curcus*). Department of Biology, Silpakon University, Thailand.