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ORIGINAL ARTICLE

Efficient plant regeneration from immature embryo cultures of *Jatropha curcas*, a biodiesel plant

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Abstract *Jatropha curcas* L. (Physic nut) is a commercially important non-edible oil seed crop known for its use as an alternate source of biodiesel. In order to investigate the morphogenic potential of immature embryo, explants from four developmental stages were cultured on medium supplemented with combinations of auxins and cytokinins. It was found that the size of embryo is critical for the establishment of callus. Immature embryos (1.1–1.5 cm) obtained from the fruits 6 weeks after pollination showed a good response of morphogenic callus induction (85.7%) and subsequent plant regeneration (70%) with the maximum number of plantlets (4.7/explant) on Murashige and Skoog's (MS) medium supplemented with IBA (0.5 mg l^{-1}) and BA (1.0 mg l^{-1}). The above medium when supplemented with growth adjuvants such as 100 mg l^{-1} casein hydrolysate + 200 mg l^{-1} L-glutamine + 8.0 mg l^{-1} $CuSO_4$ resulted in an even higher frequency of callus induction (100%). Plant regeneration (90%) with the maximum number of plantlets (10/explant) was achieved on MS medium supplemented with 500 mg l^{-1} polyvinyl pyrrolidone + 30 mg l^{-1} citric acid + 1 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 0.25 mg l^{-1} IBA. It was observed that plantlet regeneration could occur either through organogenesis of morphogenic callus or via multiplication of pre-existing meristem in immature embryos. The age of immature embryos and addition of a combination of growth adjuvants to the culture medium appear to be critical for obtaining high regeneration rates. Well-developed shoots rooted on half-strength MS medium supplemented with 0.5 mg l^{-1} IBA and 342 mg l^{-1} trehalose. The rooted plants after acclimatization were successfully transferred to the field in different agro-climatic zones in India. This protocol has been successfully evaluated on five elite lines of *J. curcas*.

Keywords Energy crop · Immature embryo · *Jatropha curcas* · Morphogenesis · Plant regeneration · Tree-borne oil seed

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	6-Benzylaminopurine
CH	Casein hydrolysate
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kn	Kinetin
MS	Murashige and Skoog basal medium
NAA	α-Naphthaleneacetic acid
PIC	4-Amino-3,5,6-tri-chloropicolinic acid
PVP	Polyvinyl pyrrolidone
WAP	Weeks after pollination

Introduction

Jatropha curcas (Euphorbiaceae) has been identified as a plant with high potential in biofuel plantations, primarily due to its relatively high seed oil content and non-competing demand with edible oil supplies. There is a growing interest in the use of *J. curcas* seed oil to alleviate the energy crisis. It is relatively simple to convert to biodiesel

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by chemical (Berchmans and Hirata 2008) or biological trans-esterification (Modi et al. 2007). According to one report, properties of biodiesel derived from *J. curcas* oil conform to American and European standards (Tiwari et al. 2007). Besides its use as a biofuel crop, it is also desired due to its drought hardiness, rapid growth, easy propagation, low cost of seeds, oil content, and small gestation period, leading to wide adoption and production in good and degraded soils (Gubitz et al. 1999; Jones and Miller 1991).

However, a major bottleneck to its adoption is the non-availability of superior clones in large numbers. Some of the traits such as higher seed yield, oil content, synchronous maturity and early flowering can be introduced to make this technology sustainable and viable. Micropropagation has been reported in *J. curcas* through both direct and callus-mediated shoot regeneration and somatic embryogenesis using epicotyls, hypocotyls, peduncles, axillary buds, nodal segments, leaf segments, and shoot tips as explant sources (Sujatha et al. 2008; Qin et al. 2004; Jha et al. 2007; Datta et al. 2007; Deore and Johnson 2008). Although extensive work has been carried out using the above explants, there is no report on the morphogenic potential of embryo explants. The potential use of immature embryos in organogenesis and genetic transformation has been demonstrated in numerous monocotyledonous species (Choi et al. 2003). However, in dicotyledons, especially of the Euphorbiaceae, there are only a few reports on plant regeneration from immature embryos. In order to explore the morphogenic potential of immature embryos from *J. curcas*, we have undertaken a comprehensive study of various factors including the age of the embryo explants. As a result, we report a protocol for high frequency plant regeneration from immature embryo cultures, which can be extended to *Agrobacterium*-mediated genetic transformation for the incorporation of useful traits.

Materials and methods

Plant material

Fruits from five different lines (R044, S002, S006, S007, and S009) of *Jatropha curcas* L. were collected from Reliance Life Sciences farm, Samalkot, Kakinada, Andhra Pradesh, India. Fruits were collected after 3, 4, 5, and 6 weeks after pollination (WAP) and classified as fruit size classes 1, 2, 3, and 4, respectively. The seed size, texture, and color were the criteria used to select the right stage of the explant. Classes 1 and 2 (3 and 4 WAP, respectively) were characterized by green fruit color and a whitish seed coat with watery endosperm. Class 3 (5 WAP) was characterized by green fruit color and a light brown seed coat with watery but occasionally viscid endosperm. For class 4 (6 WAP), dark green fruit color and dark brown to black seed coat with white, developed endosperm was typical. Fruit sizes and their respective immature embryo sizes were recorded (Table 1).

Embryo isolation

Seeds were removed from fruits with a surgical scalpel and surface-sterilized according to Deore and Johnson (2008). The seed coat was removed with the help of a sterile mortar and pestle under a laminar flow hood, and immature embryos (immature embryonal axis + cotyledons) dissected.

Callus induction medium

The immature embryos were longitudinally dissected into 6–7 small pieces and placed horizontally in 90-mm Petri plates (Greiner, Germany) containing 25 ml medium of the following composition: MS (Murashige and Skoog 1962) basal salts with 3.0% sucrose, pH 5.8 solidified with 0.75% agar (Hi-media, Mumbai) and supplemented with different concentrations of auxins (2,4-D, IAA, IBA, NAA, PIC) or

Table 1 Effect of the developmental stage of immature embryo on callus induction and subsequent plant regeneration capacity of *Jatropha curcas* in vitro

Age of the explant (WAP)[a]	Stages of physiological maturity of fruit	Size class of the fruits	Size of the fruits (cm)	Embryo size (cm)	Percent of callus induction[b]	Percent of plant regeneration[c]
3	Immature, green	1	2.4	0.2–0.3	10 (±0.5)	No
4	Immature, green	2	2.6	0.4–0.5	10 (±0.62)	No
5	Immature, green	3	2.8	0.7–0.9	30 (±1.5)	No
6	Immature, dark green	4	3.2	1.1–1.5	100 (±3.9)	70 (±2.2)

[a] Weeks after pollination

[b] Data obtained 4 weeks after culture on callus induction medium (CIM-2) consisting of MS medium with 3.0% sucrose supplemented with 100 mg l^{-1} casein hydrolysate + 200 mg l^{-1} L-glutamine + 0.5 mg l^{-1} IBA + 1.0 mg l^{-1} BA + 8.0 mg l^{-1} $CuSO_4$

[c] Data obtained 4 weeks after culture on regeneration medium (RM) consisting of MS medium + 3.0% sucrose + 500 mg l^{-1} polyvinyl pyrrolidone + 30 mg l^{-1} citric acid + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 0.25 mg l^{-1} IBA

cytokinins (BA or Kn) alone or in 36 combinations. The data on callus induction response were collected after 4 weeks of culture on callus induction medium. After identifying the most suitable medium for the callus induction (termed CIM-1), CIM-1 was supplemented with different growth adjuvants (casein hydrolysate, L-glutamine, L-proline, silver nitrate, copper sulphate) either alone or in combination (termed CIM-2). The CIM-2 arrived at consisted of MS medium with 3.0% sucrose supplemented with 100 mg l^{-1} casein hydrolysate + 200 mg l^{-1} L-glutamine + 0.5 mg l^{-1} IBA + 1.0 mg l^{-1} BA + 8.0 mg l^{-1} $CuSO_4$. After 4 weeks on CIM-2, only morphogenic calli were transferred to callus maintenance medium (CMM). The CMM consisted of MS medium with 3.0% sucrose supplemented with 100 mg l^{-1} casein hydrolysate + 200 mg l^{-1} L-glutamine + 8.0 mg l^{-1} $CuSO_4$ with reduced concentrations of IBA (0.25 mg l^{-1}).

Regeneration medium

The morphogenic calli (compact, green with shoot-bud like structures) growing on CMM were transferred to the regeneration medium for 2–3 subcultures at 3-week intervals. To study the effect of growth adjuvants on regeneration of morphogenic calli, the regeneration medium was supplemented with a number of adjuvants: copper sulphate at 8.0 mg l^{-1}, casein hydrolysate at 100 mg l^{-1}, L-glutamine at 200 mg l^{-1}, silver nitrate at 8.5 and 17.0 mg l^{-1}, and trehalose at 34.2, 171, 342, and 682 mg l^{-1}, alone or in combinations. The optimized regeneration medium was termed RM. The RM consisted of MS medium + 3.0% sucrose + 500 mg l^{-1} polyvinyl pyrrolidone + 30 mg l^{-1} citric acid + 1 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 0.25 mg l^{-1} IBA. For each medium test, 20 morphogenic callus pieces were used. The regeneration capacity was evaluated after 30 days.

Root induction

Regenerated shoots (2–4 cm in length) were transferred to 8 different media for root induction. Data on root induction was scored after 30 days. In all experiments, the pH was adjusted to 5.8 before autoclaving and the medium was solidified with 0.7% agar (Hi-media, Mumbai) and sterilized for 20 min at 121°C temperature. All the cultures were incubated at 16 h/8 h (light/dark) photoperiod cycle illuminated with cool fluorescent lamps at an intensity of 30 µmol m^{-2} s^{-1}at 24 ± 2°C.

Field transfer of the regenerated plantlets

The well-rooted regenerated plants were transferred to the soil in an environmental chamber (Sanyo, Japan) at 80% relative humidity, 24 ± 2°C temperature and 12 h photoperiod with 30 µmol m^{-2} s^{-1}at light intensity. The soil mixture contained 1:1:1:1 garden soil:coco peat:sand:vermicompost. After initial acclimatization, the plants were transferred to a polyhouse at 28–30°C. At approximately 30 cm in height, plants were transferred to the field for evaluation. Schematic representation of optimized protocol is presented in Fig. 1.

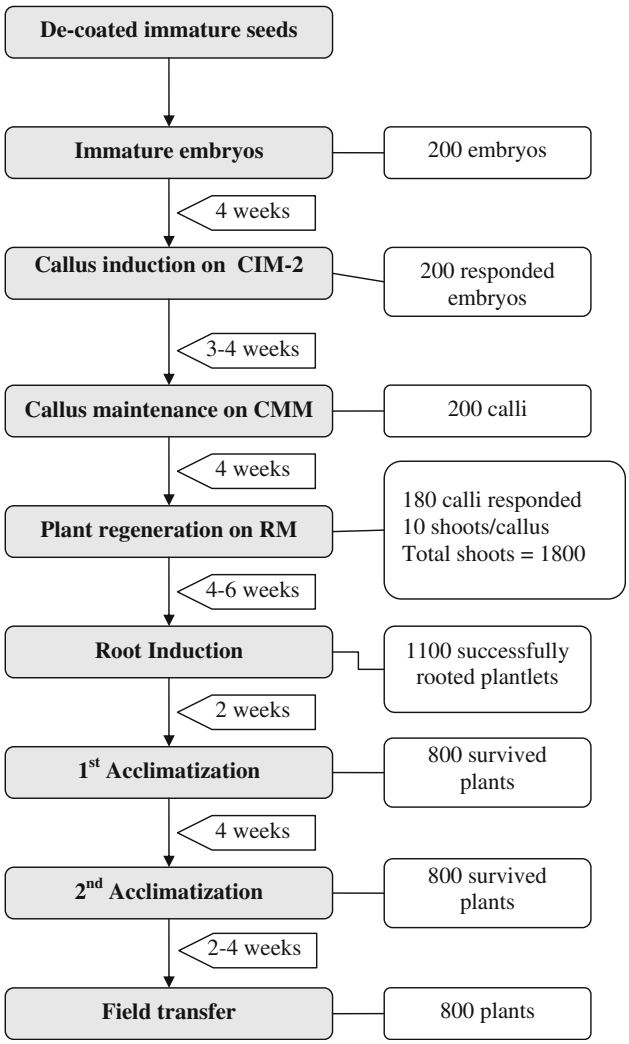

Fig. 1 Schematic representation of optimized protocol from plant regeneration to field transfer in immature embryo cultures of *Jatropha curcas*

Statistical analysis

Callus induction rate and morphological changes were recorded by visual observation and data analyzed statistically to establish SEM at 95% confidence for significant differences between treatments (Kennedy and Neville 1976). All experiments were repeated thrice with three replicates, each with 20 explants.

Results

Effect of age of the immature embryo for callus induction and plant regeneration

To determine the effect of the age of the immature embryo on callus induction, four size classes of immature embryos (0.2–0.3, 0.4–0.5, 0.7–0.9, and 1.1–1.5 cm in length) (Table 1) were cultured on MS medium supplemented with a range of hormones (2,4-D, PIC, IBA, IAA, Kn, BA, etc.). There were significant differences in the callus formation among the size classes. The immature embryo size class-4 (1.1–1.5 cm) obtained from immature fruits 6 WAP produced highest callus formation (100%) on MS medium supplemented with lower concentration of IBA (0.5 or 1.0 mg l^{-1}). The subsequent plant regeneration was also highest (70%) in the above size class (Table 1). The immature embryo size classes 1–3 (0.2–0.3, 0.4–0.5, and 0.7–0.9 cm) obtained from fruits 3, 4, and 5 WAP and embryos obtained from fruits beyond 7 WAP produced only non-morphogenic callus (friable and cream).

Optimization of callus induction medium and influence of growth adjuvants

Different concentrations and combinations of growth regulators were tested to define an efficient medium for callus induction from immature embryo cultures of *J. curcas*. Initially, different concentrations of auxins (2,4-D, IAA, IBA, NAA, and PIC) and cytokinins (BA and Kn) were used alone in supplementation to MS basal medium for the initial response of callus induction. Two types of callus induction were observed. Some of the callus was green and compact which induced plantlets (termed as morphogenic callus), but in other cases, friable and cream callus was formed that did not regenerate (non-morphogenic callus). Among auxins, it was found that IBA was most appropriate for more morphogenic callus induction. Subsequently, further experiments were carried out with IBA in combination with cytokinins BA and Kn (Table 2). Immature embryos grown on MS basal medium supplemented with IBA (0.2, 0.5 and 1 mg l^{-1}) and cytokinins BA or Kn (0.2, 0.5 and 1.0 mg l^{-1}) exhibited a wide range of callus induction percentage (14.3–85.7%). In media with

Table 2 Effect of various growth regulators on induction of callus and subsequent plant regeneration from immature embryo cultures of *Jatropha curcas*

Growth regulators (mg l^{-1})[a]		Callus formation (%)	Subsequent plant regeneration[b] (%)	Shoots per callus piece (mean)
Auxin	Cytokinin			
0.2 IBA	0.2 Kn	28.6 (±1.5)	–	2.0 (±0.12)
	0.5 Kn	28.6 (±1.8)	–	1.0 (±0.056)
	1.0 Kn	14.3 (±0.9)	–	–
0.5 IBA	0.2 Kn	14.3 (±0.95)	–	–
	0.5 Kn	14.3 (±0.93)	–	–
	1.0 Kn	28.5 (±1.75)	–	–
1.0 IBA	0.2 Kn	28.5 (±1.66)	–	–
	0.5 Kn	28.5 (±1.72)	–	–
	1.0 Kn	14.3 (±0.97)	–	–
0.2 IBA	0.2 BA	14.3 (±1.02)	–	–
	0.5 BA	28.6 (±1.75)	–	–
	1.0 BA	42.9 (±2.2)	20 (±1.34)	3.6 (±0.19)
0.5 IBA	0.2 BA	42.9 (±2.0)	25 (±1.25)	3.9 (±0.21)
	0.5 BA	57.1 (±2.71)	40 (±1.97)	2.0 (±0.13)
	1.0 BA	85.7 (±4.2)	70 (±3.5)	4.7 (±0.30)
1.0 IBA	0.2 BA	71.4 (±3.9)	0	0.0
	0.5 BA	42.9 (±2.3)	0	0.0
	1.0 BA	57.1 (±2.82)	40 (±2.01)	1.3 (+0.064)

Data scored after 4 weeks of culture

[a] Growth regulators were supplements to MS medium containing 3.0% sucrose

[b] Only green, compact and regenerating calli were transferred to regeneration medium containing MS medium + 3.0% sucrose + 500 mg l^{-1} polyvinyl pyrrolidone + 30 mg l^{-1} citric acid + 1.0 mg l^{-1} BA, 0.5 mg l^{-1} Kn + 0.25 mg l^{-1} IBA + 8.0 mg l^{-1} $CuSO_4$

Table 3 Supplementation of different growth additives to the CIM-1 and their influence on further improvement of the callus induction and subsequent plant regeneration from immature embryo cultures of *Jatropha curcas*

Treatments[a]	Callus formation (%)	Subsequent plant regeneration[b] (%)	Shoots per callus piece (mean)
100 mg l^{-1} CH	42.9 (±2.89)	30 (±1.53)	4.7 (±0.32)
200 mg l^{-1} CH	57.1 (±3.11)	50 (±2.76)	4.4 (±0.25)
500 mg l^{-1} CH	57.1 (±3.30)	60 (±3.02)	2.7 (±0.138)
100 mg l^{-1} L-glutamine	71.4 (±4.23)	50 (±2.77)	2.0 (±0.12)
200 mg l^{-1} L-glutamine	85.7 (±4.34)	45 (±2.29)	2.0 (±0.124)
500 mg l^{-1} L-glutamine	71.4 (±3.71)	70 (±3.69)	4.0 (±0.24)
100 mg l^{-1} CH + 100 mg l^{-1} L-glutamine	85.7 (±3.98)	70 (±3.68)	3.8 (±0.21)
100 mg l^{-1} CH + 200 mg l^{-1} L-glutamine	100.0 (±5.1)	90 (±4.53)	9.1 (±0.46)
100 mg l^{-1} CH + 500 mg l^{-1} L-glutamine	85.7 (±3.84)	80 (±4.00)	0.0
10 mg l^{-1} L-proline	57.1 (±3.20)	40 (±2.18)	1.0 (±0.051)
50 mg l^{-1} L-proline	57.1 (±3.31)	50 (±2.78)	1.0 (±0.052)
100 mg l^{-1} L-proline	42.9 (±2.86)	40 (±2.14)	0.0
1.7 mg l^{-1} $AgNO_3$	71.4 (±3.70)	50 (±2.81)	1.0 (±0.054)
8.5 mg l^{-1} $AgNO_3$	71.4 (±3.75)	70 (±3.64)	6.0 (±0.32)
17 mg l^{-1} $AgNO_3$	85.7 (±3.96)	80 (±4.03)	2.0 (±0.12)
0.8 mg l^{-1} $CuSO_4$	85.7 (±3.97)	60 (±3.04)	1.3 (±0.064)
8.0 mg l^{-1} $CuSO_4$	85.7 (±3.89)	90 (±4.45)	1.0 (±0.057)
16 mg l^{-1} $CuSO_4$	71.4 (±3.62)	70 (±3.65)	0.0
100 mg l^{-1} CH + 200 mg l^{-1} L-glutamine + 8.0 mg l^{-1} $CuSO_4$	100.0 (±5.2)	90 (±5.10)	10.0 (±0.5)

Data scored after 4 weeks of culture

[a] MS medium supplemented with 3.0% sucrose + 0.5 mg l^{-1} IBA + 1.0 mg l^{-1} BA was taken as a basal medium for the callus induction

[b] Only green, compact and regenerating calli were transferred to regeneration medium containing MS medium with 3.0% sucrose + 500 mg l^{-1} polyvinyl pyrrolidone (PVP) + 30 mg l^{-1} citric acid + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 0.25 mg l^{-1} IBA + 8.0 mg l^{-1} $CuSO_4$

0.5 mg l^{-1} IBA in combination with the tested concentration of BA or Kn, non-morphogenic callus along with morphogenic callus was formed. It was found that IBA (0.5 mg l^{-1}) in combination with BA (1.0 mg l^{-1}) induced the highest percentage of morphogenic, regenerable callus (85.7%). This medium was termed callus induction medium (CIM-1).

To further improve the callus induction response, CIM-1 was supplemented with growth adjuvants like L-glutamine, casein hydrolysate, L-proline, $CuSO_4$, or $AgNO_3$ alone or in combination (Table 3). Cultures grown on the media with these additives showed the callus formation in the range of 42.9% (with 100 mg l^{-1} casein hydrolysate) and 100% (with 100 mg l^{-1} casein hydrolysate + 200 mg l^{-1} L-glutamine). However, the maximum morphogenic calli (Fig. 2a, b) were found on CIM-1 medium supplemented with 100 mg l^{-1} casein hydrolysate + 200 mg l^{-1} L-glutamine, and 8.0 mg l^{-1} $CuSO_4$. Subsequent plant regeneration was also found to be highest (90%) on the above media. The rest of the growth adjuvants such as L-proline and $AgNO_3$ did not have marked influence on callus induction. Finally, the best callus induction medium arrived at consisted of MS medium supplemented with IBA (0.5 mg l^{-1}) + BA (1.0 mg l^{-1}) + casein hydrolysate (100 mg l^{-1}) + L-glutamine (200 mg l^{-1}) + $CuSO_4$ (8.0 mg l^{-1}). This medium was termed CIM-2. It was found from the present study that there was a remarkable increase in callus induction percentage and subsequent plant regeneration when CIM-1 was supplemented with a combination of growth adjuvants (Fig. 2c). The present study established a direct correlation between role of growth adjuvants and age of embryo in inducing morphogenic callus and subsequent plant regeneration.

Optimization of callus maintenance medium

For proliferation of callus, optimum CMM was defined after testing 36 different combinations of growth regulators. The CMM consisted of MS medium supplemented with 100 mg l^{-1} casein hydrolysate + 200 mg l^{-1} L-glutamine + 8.0 mg l^{-1} $CuSO_4$ with reduced concentrations of IBA (0.25 mg l^{-1}).

Optimization of regeneration medium and influence of growth adjuvants

The RM developed by our group (Deore and Johnson 2008) was taken as a reference medium for present investigation.

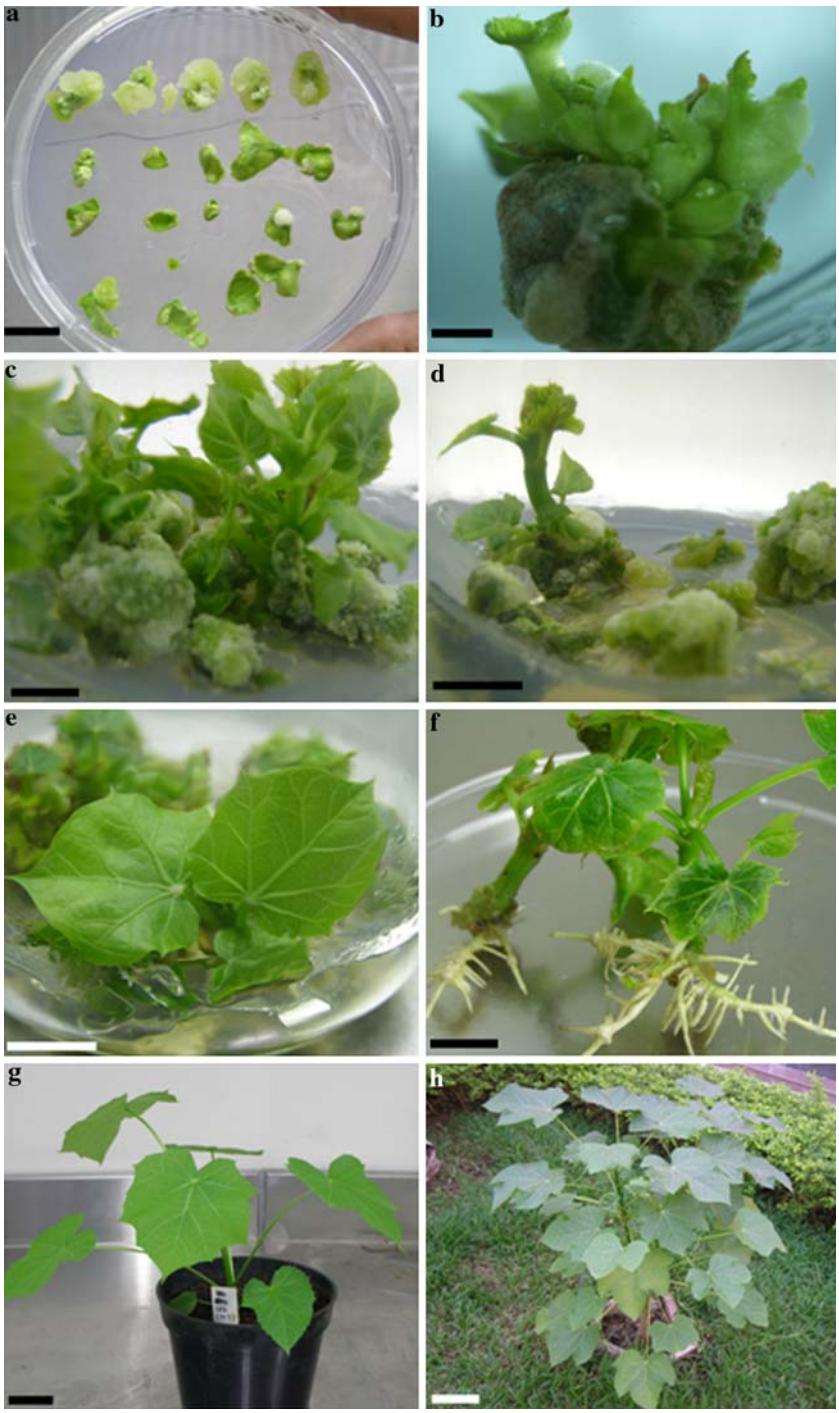

Fig. 2 Callus induction and plant regeneration from immature embryo cultures of *J. curcas*. **a** Induction of callus from immature embryos (*bar* 1 cm). **b** Morphogenic callus showing induction of shoot buds (*bar* 1 mm). **c** Subsequent plant regeneration from shoot buds (*bar* 1 cm). **d**,**e** Elongation of regenerated plantlets (*bar* 1 cm). **f** Induction of roots from regenerated plantlets (*bar* 1 cm). **g** Acclimatization of regenerated plants in green house (*bar* 3 cm). **h** Completely established plant growing in field conditions (*bar* 10 cm)

In order to improve regeneration capacity of calli, the above medium was supplemented with growth adjuvants such as trehalose, $CuSO_4$, casein hydrolysate, and L-glutamine. None of the tested growth adjuvants have any significant influence on plant regeneration except medium supplemented with $CuSO_4$ (8.0 mg l^{-1}) which yielded more than 4 shoots per callus piece (Table 4). The RM arrived at consisted of MS medium supplemented with 500 mg l^{-1} PVP + 30 mg l^{-1} citric acid + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 0.25 mg l^{-1} IBA + 0.5 mg l^{-1} GA_3 + 8.0 mg l^{-1} $CuSO_4$.

The morphogenic callus from immature embryos was initiated and maintained for the further proliferation on CIM-2 and CMM, respectively for total period of 8 weeks. Upon transfer to RM, two types of shoot regeneration were observed, i.e. healthy single shoot

Table 4 Optimization of regeneration medium for plantlet regeneration from callus obtained from immature embryo cultures of *Jatropha curcas*

Treatment	Responded explant (%)	Regenerated shoots per callus piece
Base medium (BM)[a]	60 (±3.20)	S[c]
BM + 34.2 mg l^{-1} trehalose	30 (±1.71)	S[b]
BM + 171 mg l^{-1} trehalose	40 (±2.01)	S[b]
BM + 342 mg l^{-1} trehalose	30 (±1.65)	S[b]
BM + 682 mg l^{-1} trehalose	40 (±2.12)	S[b]
BM + 8.0 mg l^{-1} $CuSO_4$	90 (±4.57)	S[d]
BM + 100 mg l^{-1} CH + 200 mg l^{-1} L-glutamine	10 (±0.54)	S[b]
MS basal salt + 3.0% sucrose + 0.125 mg l^{-1} IBA + 0.125 mg l^{-1} IAA + 0.5 mg l^{-1} GA_3	50 (±2.82)	S[c]
MS basal salt + 3.0% sucrose + 0.125 mg l^{-1} IBA + 0.125 mg l^{-1} IAA + 0.5 mg l^{-1} GA_3 + 8.5 mg l^{-1} $AgNO_3$	60 (±3.31)	S[c]

Data recorded after 4 weeks of culture

[a] Base medium (BM) contained MS medium + 3.0% sucrose + 500 mg l^{-1} polyvinyl pyrrolidone (PVP) + 30 mg l^{-1} citric acid + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 0.25 mg l^{-1} IBA

[b] 1 shoot per callus

[c] 2–4 shoots per callus

[d] More than 4 shoots per callus

Table 5 Effect of different growth regulators on root induction in regenerated shoots of *Jatropha curcas*

Growth regulators[a]	Root induction response		No. of roots per plant
	Type	Percent	
0.5 mg l^{-1} IBA	PR	20 (±1.09)	2
0.5 mg l^{-1} IBA + 0.5 mg l^{-1} Zeatin + 0.5 mg l^{-1} GA_3	–	–	–
0.5 mg l^{-1} GA_3	–	–	–
0.5 mg l^{-1} Zeatin	–	–	–
0.5 mg l^{-1} IAA + 0.5 mg l^{-1} GA_3	–	–	–
0.5 mg l^{-1} NAA	–	–	–
0.5 mg l^{-1} IBA + 342 mg l^{-1} Trehalose	PR and SR	80 (±3.89)	>4

Data scored after 4 weeks of culture

PR Primary roots, *SR* secondary roots

[a] Half-MS medium with 2.0% sucrose has taken as basal medium for rooting experiments

regeneration from morphogenic callus through organogenesis (Fig. 2d, e) and multiple shoot regeneration through multiplication of pre-existing apical meristems of immature embryo. However, frequency of shoot regeneration of pre-existing apical meristem was less compared to organogenesis.

Root induction

The regenerated shoots (2–4 cm in length) were transferred to half-MS medium with 2.0% sucrose and supplemented with different concentrations of zeatin or NAA or IAA or IBA with or without trehalose (Table 5). Root formation was observed on medium supplemented either with IBA (0.5 mg l^{-1}) alone in 20–30% cultures or IBA (0.5 mg l^{-1}) in combination with 342 mg l^{-1} trehalose in 60–80% cultures (Fig. 2f). The best result was found on half-MS medium with 2.0% sucrose and supplemented with 0.5 mg l^{-1} IBA and 342 mg l^{-1} trehalose (Table 5).

Field transfer of regenerated plants

The well-developed regenerated plantlets with 4- to 5-cm-long shoots and 5- to 9-cm-long primary roots with secondary branches were transferred to the soil for the first acclimatization. The acclimatized plantlets were transferred to the polyhouse after 2 weeks (Fig. 2g) and when these plants attained 24–30 cm in height they were

Table 6 In vitro culture response of immature embryo cultures of five elite lines of *Jatropha curcas*—reproducibility of optimized protocol for callus induction and subsequent plant regeneration

Name of the line	Size of the fruits (cm)	Age of the explant (WAP)	Embryo size including cotyledon (cm)	Response for callus induction[a]	Subsequent plant regeneration (%)[b]
R 044	3.4	6	1.1–1.5	100 (±4.75)	90 (±3.9)
S 002	3.1	6	1.2–1.4	70 (±2.75)	70 (±2.86)
S 006	3.2	6	1.2–1.5	70 (±2.82)	65 (±2.40)
S 007	3.2	6	1.1–1.5	95 (±4.01)	90 (±3.76)
S 009	3.1	6	1.0–1.4	90 (±3.96)	90 (±3.84)

Data scored after 4 weeks of culture

[a] CIM-2 consisting of MS medium with 3.0% sucrose supplemented with 100 mg l^{-1} casein hydrolysate + 200 mg l^{-1} glutamine + 0.5 mg l^{-1} IBA + 1.0 mg l^{-1} BA + 8.0 mg l^{-1} $CuSO_4$ was used for callus induction

[b] Regeneration medium consisting of MS medium + 3.0% sucrose + 500 mg l^{-1} polyvinyl pyrrolidone + 30 mg l^{-1} citric acid + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 0.25 mg l^{-1} IBA + 8.0 mg l^{-1} $CuSo_4$ was used for plant regeneration

transferred to the field in different agro-climatic zones of India (Fig. 2h). No apparent phenotypic variations were observed in regenerated plants.

Evaluation of developed protocol on other genotypes

The results were successfully reproduced when the developed protocol was tested on other elite lines (S 002, S 006, S 007, and S 009) of *J. curcas* (Table 6).

Discussion

The main objective of this study was to establish a reproducible regeneration system in *J. curcas* using immature embryo cultures. Immature embryos are the most responsive source to regenerate plantlets among other explants in culture. The developmental stage of the explant is reported to be a crucial factor for in vitro regeneration in cereal crops (He et al. 1988). In this study, it was found that the optimum developmental stage and size of immature embryos of *J. curcas* for callus induction and subsequent plant regeneration was size class 4, 1.1–1.5 cm in length, obtained from immature fruit 6 WAP. Induction of callus was the critical stage in tissue culture where the quality and type of callus influences subsequent plant regeneration. So far, to the best of our knowledge, no comprehensive study has been undertaken to study the relationship between morphogenic potential and the developmental stage of embryo in *J. curcas*. Our study revealed that the developmental stage of the immature embryo is critical for maximum morphogenic response.

Plant growth regulators, especially cytokinins and auxins alone and in combination, are known to play a very important role in the process of callus induction and its proliferation. However, the optimum concentrations for inducing callus differ according to the type of auxin, genotype, and explant source. In the present study, IBA in combination with Kn induced a lower percentage of callus while, in combination with BA, promoted the highest percentage of callus induction (85.7%), maximum subsequent plant regeneration (70%), and highest number of shoots per explant (4.7) (Table 2). BA has been reported to be more beneficial than other cytokinins for micropropagation of various members of the Euphorbiaceae (Tideman and Hawker 1982; Ripley and Preece 1986). In *Jatropha integerrima*, a high frequency callus induction was reported in media containing IBA + BA in hypocotyls, stem, peduncle, and leaf explants (Sujatha et al. 2008). Similarly, in *J. curcas*, BA promoted higher regeneration from hypocotyls and petiole explants than kinetin (Sujatha and Mukta 1996). The favorable influence of BA on the morphogenic capacity of the explants has also been reported in other species: castor (Sarvesh et al. 1992; Sujatha et al. 2008), *Euphorbia peplus* (Tideman and Hawker 1982), and *E. hirta* (Baburaj et al. 1987). But all these studies were on explants other than immature embryos. Our data on morphogenic callus induction in immature embryo cultures of *J. curcas* showed very similar observations of the favorable influence of BA. The present results indicate the efficiency of the IBA–BA combination on the induction of callus (Table 2). The differential response of cytokinins, i.e. in this case, BA and Kn, may be attributed to differences in uptake, levels of endogenous growth regulators, and recognition by cells. Induction of callus is a critical stage where subsequent plant regeneration is highly dependent and governed by growth regulators used during the callus induction stage.

After determining the optimum combination of IBA and BA (CIM-1), the incorporation of growth adjuvants, such as L-proline, L-glutamine, casein hydrolysate, $CuSO_4$, or $AgNO_3$ to further enhance induction of morphogenic callus and subsequent plant regeneration, was studied. It has been reported that the rate of plant regeneration increases with

the addition of casein hydrolysate and amino acids such as L-glutamine and L-proline (Minhas et al. 1999). The present study showed that supplementation of L-glutamine (200 mg l^{-1}) alone to CIM-1 induced callus in 85.7% explants but subsequent plant regeneration was less. Casein hydrolysate supplementation individually induced callus induction in 42.9–57.1% of explants depending on the concentration used, while the number of shoots produced per explant varied from 2.7 to 4.7 (Table 3). But when casein hydrolysate and L-glutamine were used in combination, there was callus induction in 100% explants, subsequent plant regeneration in 90% explants and 9.1 shoots produced per explant, indicating synergistic effect of both additives.

$AgNO_3$ is a potent inhibitor of ethylene action, and ethylene is considered to suppress shoot organogenesis in vitro. In the present study, supplementing $AgNO_3$ to CIM-1 resulted in increase in shoot regeneration (80%) and increase in number of shoots produced per explant (up to 6) from immature embryo cultures of *J. curcas*. Increase in copper concentration was also found to enhance the regeneration frequency (Nirwan and Kothari 2003). In the present investigation, addition of copper in the form of $CuSO_4$ to CIM-1, although increased callus induction and subsequent plant regeneration but number of shoots produced per explant were very low (Table 3). However, supplementation of CIM-1 with casein hydrolysate, glutamine, and $CuSO_4$ together produced the best results, achieving 100% callus induction, 90% subsequent plant regeneration, and up to 10 shoots per explant. The synergistic effect of a combination of growth adjuvants and the developmental stage of the immature embryo appear to play a critical role in obtaining increased plant regeneration as is evident from this study.

Several protocols for plant regeneration are reported with varying efficiency in *J. curcas.* Li et al. (2008) claimed 33–35% shoot regeneration in cotyledon explants while Jha et al. (2007) reported 56% regeneration from leaf explants. Sujatha and Prabhakaran (2003) reported 71.2% adventitious shoot regeneration from leaf explants. Sujatha et al. (2005) claimed 79% adventitious shoot regeneration in axillary nodes and leaf sections. In the present investigation, we report 90% plant regeneration in immature embryo explants, which is so far, to our knowledge, superior to published protocols. In the present study, it was also found that the mode of plant regeneration was of two types, one is via organogenesis of the morphogenic callus which is the subject of our study and the second type is via multiplication of pre-existing mersitems in immature embryo explants. Almost similar observations were described in Hungarian vetch by Sancak et al. (2000).

Among tree-borne oil seeds, *J. curcas* emerges as the most promising feedstock for biodiesel following its successfully testing as a jet fuel by the aviation industry (Biello 2008). In spite of some doubts and criticism regarding this species, it appears to hold promise for the future. In order to be environmentally sustainable and not compete with existing food sources, it needs to be more productive (Gressel 2008). At present, high yields are achieved on good soils with relatively high inputs. The promise for commercial production on marginal lands is yet to be fulfilled. Ethanol and other biofuel crops such as corn and rapeseed have come under increasing scrutiny as they have been blamed for contributing to high food prices and deforestation. *Jatropha* being a non-edible crop with multiple utilities can be a very good alternative crop for biofuel production. Biofuel crops such as *Jatropha* would only be cost-effective in the long run when they are modified transgenically to omit toxins, suppress phorbol ester and environmental contaminants, and achieve higher production levels. For a plant to be economically viable, integrated approaches in the area of morphogenic, genomic, and agronomic are needed. The present study describes a non-genotype-specific, efficient protocol for shoot morphogenesis which is a pre-requisite for a successful genetic transformation and plant improvement. Further, technology such as this is an attempt to meet challenges and contribute to understand this hitherto little-known shrub.

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References

Baburaj S, Dhamotharan R, Santhaguru K (1987) Regeneration in leaf callus cultures of *Euphorbia hirta* Linn. Curr Sci 56:194

Berchmans HJ, Hirata S (2008) Biodiesel production from crude *Jatropha curcas* L. seed oil with a high content of free fatty acids. Bioresour Technol 99:1716–1721

Biello D (2008) Biofuel for jumbo jets: Kiwis take to the sky on Jatropha. Sci Am Dec 30, 2008

Choi P-S, Cho D-Y, Soh W-Y (2003) Plant regeneration from immature embryo cultures of *Vigna unguiculata*. Biol Plant 47:305–308

Datta MM, Mukherjee P, Ghosh B, Jha TB (2007) In vitro clonal propagation of biodiesel plant (*Jatropha curcas* L.) Curr Sci 93:1438–1442

Deore AC, Johnson TS (2008) High-frequency plant regeneration from leaf-disc cultures of *Jatropha curcas* L.: an important biodiesel plant. Plant Biotechnol Rep 2:7–11

Gressel J (2008) Transgenics are imperative for biofuel crops. Plant Sci 174:246–263

Gubitz GM, Mittelbach M, Trabi M (1999) Exploitation of the tropical oil seed plant *Jatropha curcas* L. Bioresour Technol 67:73–82

He DG, Yang YM, Scott KJ (1988) A comparison of scutellum callus and epiblast callus induction in wheat: the effect of genotype, embryo age and medium. Plant Sci 57:225–233

Jha TB, Mukherjee P, Datta MM (2007) Somatic embryogenesis in *Jatropha curcas* Linn. an important biofuel plant. Plant Biotech Rep 1:135–140

Jones N, Miller JH (1991) *Jatropha curcas*—a multipurpose species for problematic sites. Land Resour Ser 1:1–12

Kennedy JB, Neville AM (1976) Basic statistical methods for engineers and scientists, Harper International, New York

Li M, Li H, Jiang H, Pan X, Wu G (2008) Establishment of an *Agrobacterium*-mediated cotyledon disc transformation method for *Jatropha curcas*. Plant Cell Tissue Organ Cult 92:173–181

Minhas D, Rajam MV, Grover A (1999) Maintenance of callus growth during sub-culturing is a genotype dependant response in rice: mature seed-derived callus from IR-54 rice cultivar lacks culture ability. Curr Sci 77:1410–1413

Modi MK, Reddy JR, Rao BV, Prasad RB (2007) Lipase-mediated conversion of vegetable oils into biodiesel using ethyl acetate as acyl acceptor. Bioresour Technol 98:1260–1264

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Nirwan RS, Kothari SL (2003) High copper levels improve callus induction and plant regeneration in *Sorghum bicolor* (L.) Moench. In Vitro Cell Dev Biol Plant 39:161–164

Qin W, Lu Wei-Da, Yi L, Shu-lin P, Ying X, Lin T, Fang C (2004) Plant regeneration from epicotyl explant of *Jatropha curcas*. J Plant Physiol Mol Biol 30:475–478

Ripley KP, Preece JE (1986) Micropropagation of *Euphorbia lathyris* L. Plant Cell Tissue Organ Cult 5:213–218

Sancak C, Mirici S, Ozcan S (2000) High frequency shoot regeneration from immature embryo explants of *Hungarian vetch*. Plant Cell Tissue Organ Cult 61:231–235

Sarvesh A, Rao DM, Reddy TP (1992) Callus initiation and plantlet regeneration from epicotyl and cotyledonary explants of castor (*Ricinus communis* L.). Adv Plant Sci 5:124–128

Sujatha M, Mukta N (1996) Morphogenesis and plant regeneration from tissue cultures of *Jatropha curcas* L. Plant Cell Tissue Organ Cult 44:135–141

Sujatha M, Prabhakaran AJ (2003) New ornamental *Jatropha* hybrids through interspecific hybridization. Genet Resour Crop Evol 50:75–82

Sujatha M, Makkar HPS, Becker K (2005) Shoot bud proliferation from axillary nodes and leaf sections of non-toxic *Jatropha curcas* L. Plant Growth Reg 47:83–90

Sujatha M, Reddy TP, Mahasi MJ (2008) Role of biotechnological interventions in the improvement of castor (*Ricinus communis* L.) and *Jatropha curcas* L. Biotech Adv 26:424–435

Tideman J, Hawker JS (1982) In vitro propagation of latex producing plants. Ann Bot 49:273–279

Tiwari AK, Kumar A, Raheman H (2007) Biodiesel production from *Jatropha* (*Jatropha curcas*) with high free fatty acids: an optimized process. Biomass Bioenergy 31:569–575