Indian Journal of Biotechnology
Vol 11, July 2012, pp 280-287

In vitro regeneration from node and leaf explants of *Jatropha curcas* L. and evaluation of genetic fidelity through RAPD markers

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Received 17 March 2011; revised 12 July 2011; accepted 30 October 2011

Plantlet regeneration in *Jatropha curcas* L. (Family: Euphorbiaceae), an energy shrub, has been studied from nodal and leaf explants on basal MS medium. The nodal explants were found superior to leaf explants. Higher shoot bud differentiation was observed on MS media supplemented with 8 µ*M* N^6-benzyladenine (BA; 6.2±0.83) from nodal explants in comparison to both 10 µ*M* kinetin (Kn; 2.8±0.45) from leaf explants and a combination of 6.0 µ*M* BA with 4.0 µ*M* Kn (5.0±0.71) from nodal explants. MS medium fortified with 8 µ*M* BA and 2 µ*M* IBA (indole butyric acid) was found most suitable for both callus mediated organogenesis and elongation of shoots. Addition of 45 µ*M* adenine sulphate, 15 µ*M* glutamine and 10 µ*M* proline to this optimized MS medium enhanced the number of multiple shoot proliferation (9.8±0.84) per explant and elongation at the end of 2nd wk. The elongated shoots were successfully rooted on half-strength MS medium with a prior incubation on MS medium with NAA (α-napthalene acetic acid) or IBA or a combination of both; 2 µ*M* IBA provided better response for rhizogenesis among them. Regenerated plantlets were successfully established in soil where 80±4% of them developed into morphologically normal and fertile plants. Forty RAPD decamer primers were used to assess genetic fidelity of regenerated plantlets along with the donor plant. Fourteen primers responded for amplification, generating 75 amplified products (160 to 2690 bp). The amplification pattern confirmed the genetic uniformity of the regenerated plantlets and substantiated the efficacy and suitability of this protocol for *in vitro* propagation of *J. curcas*, thereby favouring the economics of the cost of plant material and time factor.

Keywords: Genetic fidelity, organogenesis, purging nut, RAPD, shoot bud differentiation

Introduction

Jatropha curcas L. (Family: Euphorbiaceae) is a shrub, commonly known as Ratanjot, Purging nut or Physic nut. This is a multipurpose, non-edible, oil yielding, perennial shrub that grows well in the tropical and subtropical climates and possesses with several medicinal properties. The plant parts including leaves, stems, seeds, roots and seed extracts have been extensively used in traditional system of medicine for various ailments, such as, pyorrhea, sciatica, paralysis, dropsy, rheumatism, cutaneous diseases, piles, fistula, scabies, eczema, ringworm, chronic dysentery, ulcer and urinary discharges[1-5]. The plant is known to possess anticancer[6], antihelmintic[7], antiparasitic, anticoagulant and purgative properties[8]. In addition, the seeds of *J. curcas* contain viscous oil, which can be used for manufacture of candles and soaps, in cosmetics industry, as a paraffin substitute or extender in automobile industry[3]. Moreover '*J. curcas* oil' can be easily processed to replace partially or fully petroleum based diesel fuel[9]. Thus, the use of this plant for large-scale biodiesel production is of great interest with regards to solving the energy shortage, reducing carbon emission and increasing the income of farmers in addition to its use in traditional medicines.

J. curcas has assumed as potential biofuel crop because of its short reproductive period, drought tolerance, low cost of seeds, high oil content, easy adaptation on marginal and semi-marginal lands, suitability as fuel substitute without any alteration to the existing engines and above all the plant size makes seed collection more convenient[10]. The requirement of *J. curcas* is presently obtained from the natural populations. To meet the large scale demand and ensure easy supply of this elite material, there is a need to establish clonal seed orchards and develop mass multiplication techniques. *J. curcas* is the most primitive species of the genus and forms artificial and natural hybrid complexes readily, which

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poses a problem to the genetic variation[10,11]. Mass propagation through stem cutting is possible but seed yields are low and the established plants are not deep rooted[12,13], hence are easily uprooted. This is a major constraint for the establishment of plants propagated through stem cuttings on poor and marginal soils. Tissue culture technology can provide an alternative way to substantiate the rapid multiplication of elite clone for continuous supply of plant material and germplasm conservation[14]. There are reports on *in vitro* propagation of *J. curcas* through direct shoot regeneration from different explants including node, shoot tip, cotyledon, hypocotyls, epicotyl[3,4,12,15-17], axillary bud proliferation[12,13] and somatic embryogenesis[18]. However, in these reports *in vitro* multiplication rate was low and the genetic fidelity of regenerated plantlets was not assessed either at cell or molecular level. The improvement of micropropagation efficiency and genetic uniformity of the plantlet is desirable to meet the need of elite clone of *J. curcas*.

In tissue culture, genetic variability often arises as a manifestation of epigenetic influence or changes in the genome of differentiating somatic cells induced by tissue culture conditions[19,20]. Therefore, genetic stability of *in vitro* regenerated plantlets has always been assessed during standardization of an efficient *in vitro* propagation protocol. Recently, molecular markers have been utilized in the detection of variation or confirmation of genetic fidelity during micropropagation[21]. Randomly amplified polymorphic DNA (RAPD) based detection of genetic polymorphism[22] is a kind of molecular marker based on DNA sequence and it has been successfully utilized to determine the clonal fidelity of micropropagated plants in several species[21,23-25].

In the present study, authors have developed an efficient and reproducible plant regeneration protocol for *J. curcas* by optimizing culture environment and determined the genetic stability of *in vitro* regenerated plantlets using RAPD markers.

Materials and Methods

Plant Materials and Explant Preparation

Fresh young leaves and nodes were excised from 3-yr-old *J. curcas* clone MITJC-11 growing in the experimental garden of Sambalpur University, Orissa, India. This clone was a selection from MITS Biodiesel Project plantation materials and it possessed with ~42% oil content. The explants were washed thoroughly under running tap water for 5 min, immersed in 2% Teepol (Merck, India) for 2-3 min and washed thoroughly with double distilled water (ddH_2O). Subsequently, the explants including leaves and nodes were surface sterilized using 0.1% $HgCl_2$ for 1 and 3 min, respectively. Surface sterilized explants were rinsed thoroughly 4 to 5 times (3 min each) using sterile ddH_2O to remove the traces.

Culture Medium and Conditions

The MS[26] culture medium was used with 555 µ*M* meso-inositol (Hi-media, India) and 3% (w/v) sucrose (Hi-media, India). The medium was augmented with different concentrations and combinations of N^6-benzyladenine (BA), kinetin (Kn), 2,4-dichlorophenoxy acetic acid (2,4-D), indole butyric acid (IBA) and α-napthalene acetic acid (NAA) (Hi-media, India). The *p*H of the medium was adjusted to 5.8 prior to gelling it with 0.8% (w/v) agar-agar (Bacteriological Grade, Hi-media, India). All the media contained in culture vessels (25×150 mm^2 culture tube and 150 mL conical flask containing 15 and 30 mL medium, respectively) were autoclaved at 104 kpa and 121°C for 20 min. One explant (~25 mm^2) was cultured in each tube/flask and cultures were incubated at 25±1°C, 60-70% relative humidity and 16 h photoperiod of 35 µE m^{-2} s^{-1} irradiance level provided by cool white fluorescent tubes (CG, India).

Multiple Shoot Induction and Shoot Elongation

The experiment was designed to study the effect of different concentrations and combinations of cytokinins (BA & Kn) and auxins (IBA & 2,4-D) on shoot bud regeneration and multiple shoot induction. Along with the growth hormones, additives like adenine sulphate (15, 30, 45, 60 µ*M*), glutamine (7.5, 15, 22.5, 30 µ*M*) and proline (5, 10, 15, 20 µ*M*) were also used to enhance the rate of multiple shoot induction and rate of elongation. After 8 wk of culture, the number of shoot buds or shoot initials per explant was counted. Each treatment was replicated 5 times using 5 explants for each treatment.

Rooting of Elongated Shoots and Acclimatization

Elongated shoots with 5-6 leaves (>3 cm long) were excised *in vitro* from all treatments and were transferred to half-strength MS medium with 2% sucrose (*p*H 5.8) containing NAA (0.4, 1, 2, 3 µ*M*) and IBA (0.4, 1, 2, 3 µ*M*) individually or in combination and incubated for overnight. Later, the treated shoots were grown on PGR free haf-strength

MS medium. Each treatment was replicated 5 times using single plantlet for each treatment. Plantlets with well-developed roots were transferred to plastic cups containing autoclaved sand and soil (1:1) and maintained in the same environmental condition for 1 wk. The plantlets were watered regularly with 1/10th strength MS liquid medium. Subsequently, plantlets were transferred to earthen pots containing coarse sand, compost and garden soil (1:1:2) and kept in shade for 2 wk before transfer to the experimental garden.

Observation and Statistical Analysis

Visual observations were made every week and data on number of shoots per explant, effect of additives on shoot regeneration frequency and number of roots per shoot was recorded at the end of 10th, 8th and 3rd wk, respectively. Each treatment consisted of 5 replicates and each experiment was repeated thrice. Data on shoot bud regeneration, multiple shoot production and rooting were statistically analyzed using completely randomized block design and means were evaluated at p=0.05 significance level using Duncan's multiple range test[27]. For this SPSS V 8.0.1 software was used with parameters of one way ANOVA and homogeneity of variance.

RAPD Marker Analysis

Genomic DNA was isolated from fresh and young leaf samples (~1.2 g) of 11 *in vitro*-regenerated plantlets of different replications as well from donor plant by using SDS method[28] with few modifications[24]. DNA was dissolved in 10 m*M* Tris/1m*M* EDTA ($T_{10}E_1$) buffer. DNA concentration and purity was measured by using UV-Vis spectrophotometer (UV 1601, Shimadzu, Japan) with $T_{10}E_1$ buffer (*p*H 8) as blank. For further confirmation the quantification of DNA was accomplished by analyzing the purified DNA on 0.8% agarose gel along with diluted uncut λ DNA as standard. DNA from all 11 regenrated plantlets and donor plant were equilibrated to concentration of 10 ng/µL using $T_{10}E_1$ buffer.

For RAPD analysis, PCR amplification of 30 ng genomic DNA was carried out using 40 standard decamer oligonucleotide primers (OPA01-20 and OPB01-20) (Operon Tech., Alameda, CA, USA) as described by William *et al*[22]. Each amplification reaction mix of 25 µL contained the 30 ng template DNA, 2.5 µL of 10× assay buffer (100 m*M* Tris Cl, *p*H 8.3; 0.5 *M* KCl; 0.1% gelatin), 2 m*M* $MgCl_2$, 200 µ*M* each of the dNTPs, 20 ng primer, 1 U Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The amplification was performed in a thermal cycler (TC-312, Techne, UK) programmed for initial denaturation of 5 min at 94°C; 45 cycles of 2 min denaturation at 94°C, 1 min annealing at 37°C and 2 min elongation at 72°C, and final elongation step at 72°C for 5 min.

The PCR products were separated on 1.4% agarose gel containing ethidium bromide solution (0.5 µg/mL of gel solutions) in submarine electrophoresis apparatus (Biotech, Yercaud) using TAE (40 m*M* Tris acetate; 2 m*M* EDTA) buffer at constant 50 V for about 3 h. A gel loading buffer (20% sucrose, 0.1 *M* EDTA, 1% SDS, 0.25% bromophenol blue, 0.25% xylene cyanol) was used as tracking dye. Amplified DNA fragments were visualized by UV transilluminator (M-15, UVP, Upland, CA 91786, USA) and documented using photostation compact (Lark Innovatives, India). The size of the amplicons was determined using 250 bp DNA ladder (Bangalore Genei Pvt. Ltd., Bangalore, India) as standard and Total Lab Solutions-V 2003.02 software. To test the reproducibility the reactions were repeated twice.

Results and Discussion

Organogenesis *in vitro* is a complex process and it depends upon the addition of exogenous plant growth regulators in the media to make the explant organogenic competent for differentiation, which varies from species to species. In the present study, complete plantlets with well developed shoots and roots were produced from nodes of *J. curcas* through direct adventitious shoot regeneration as well as through callus-mediated organogenesis.

Shoot Regeneration and Elongation

The response of leaf and node explants to various concentrations and combinations of BA and Kn in MS culture medium was studied. Direct shoot bud differentiation was observed as protrusions at the leaf disc and node segments (Fig. 1a) after 2 wk of culture initiation. By the end of 5th wk, these protruded structures developed into shoot buds without intervening callus phase and 40-50% of them were flanked by green leaves (Fig. 1b). By the end of 10th wk, well developed multiple shoots were initiated from both the explants. However, the frequency of shoot formation was influenced by the type of the explant, choice of the cytokinin and its dosage. Nodal explants cultured on MS medium without any cytokinin responded very slowly giving rise to one

Fig. 1 (a-d)—Plant regeneration in *J. curcas* L. through direct organogenesis: Protuberances at nodal explant (a), multiple shoot initials after 5 wk on MS+8.0 µ*M* BA (b), well developed multiple shoots after 10 wk from nodal explants on MS+8.0 µ*M* BA (c), & multiple shoot bud regeneration from leaf explants after 10 wk on MS+8.0 µ*M* BA+4.0 µ*M* Kn (d).

plant, while the leaf explant did not respond at all. The highest frequency of shoot formation (6.2±0.83) was observed from nodal explants on MS medium with 8 µ*M* BA (Table 1; Fig. 1c). However, in case of leaf explants, a maximum of only 2.8±0.45 shoots developed on MS medium with 8 µ*M* BA and 4 µ*M* Kn (Fig. 1d). Thus, nodal explants were found superior to leaf explants. Moreover, of the two cytokinins used, BA was found more effective than Kn in inducing multiple shoots, indicating cytokinin specificity for multiple shoot induction in *J. curcas*. Similar kind of report was also made previously in other species[29]. Datta *et al*[13] have also reported that MS medium fortified with 22.2 µ*M* BA and 55.6 µ*M* adenine sulfate regenerated shoot buds (6.2 ± 0.83) from nodal explants of *J. curcas*. But MS medium fortified with 2.3 µ*M* Kn, 0.5 µ*M* IBA and 27.8 µ*M* adenine sulfate was found more effective in term of number of shoots per explant (30.8 ± 5.48)[13]. It was also reported that MS medium enriched with 6.66 µ*M* BA, 2.32 µ*M* Kn and 0.285 µ*M* indole acetic acid (IAA) regenerated even more (45) shoot buds per explant[16]. Addition of IBA along with BA has also been reported to regenerate shoot buds from the nodal explants[17,30]. However, in the present study, addition of IBA along with BA promoted callus mediated

Table 1—Effect of different cytokinins (BA & Kn) on development of multiple shoots of *J. curcas* from two different explants (nodal and leaf) after 10 wk

MS medium with cytokinin (µ*M*)		No. of shoots per explant (mean±SD)*	
BA	Kn	Node	Leaf
Control		1.2±0.45 ^{ab}	0.0±0.00 ^{a}
2.0	-	1.2±0.45 ^{ab}	0.0±0.00 ^{a}
4.0	-	1.8±0.45 ^{abcd}	0.4±0.55 ^{ab}
6.0	-	3.2±0.83 ^{ghi}	1.2±0.45 ^{cde}
8.0	-	6.2±0.83 ^{k}	1.2±0.45 ^{cde}
10.0	-	3.6±0.55 ^{hi}	1.8±0.45 ^{ef}
-	2.0	1.0±0.00 ^{a}	0.4±0.55 ^{ab}
-	4.0	1.6±0.55 ^{abc}	0.4±0.55 ^{ab}
-	6.0	1.6±0.55 ^{abc}	0.6±0.55 ^{abc}
-	8.0	2.4±055 ^{cdefg}	1.4±0.55 ^{def}
-	10.0	2.8±0.45 ^{efgh}	1.8±0.45 ^{ef}
2.0	2.0	1.8±0.45 ^{abcd}	1.0±0.00 ^{bcd}
2.0	4.0	2.2±0.83 ^{cdef}	1.4±0.55 ^{def}
2.0	6.0	2.4±0.55 ^{cdefg}	1.6±0.55 ^{def}
4.0	2.0	1.6±0.55 ^{abc}	1.4±0.55 ^{def}
4.0	4.0	2.8±0.45 ^{efgh}	1.6±0.55 ^{def}
4.0	6.0	2.6±0.55 ^{defg}	1.6±0.55 ^{def}
6.0	2.0	4.0±0.71 ^{i}	1.8±0.45 ^{ef}
6.0	4.0	5.0±0.71 ^{j}	2.0±0.71 ^{fg}
6.0	6.0	2.0±0.71 ^{bcde}	0.6±0.55 ^{abc}
8.0	2.0	3.6±1.14 ^{hi}	1.4±0.55 ^{def}
8.0	4.0	3.0±0.71 ^{fgh}	2.8±0.45 ^{h}
8.0	6.0	2.8±0.45 ^{efgh}	2.6±0.55 ^{gh}

*****Means within a column having the same letter are not statistically significant (p=0.05) according to Duncan's multiple range test (SPSS V 8.0.1)

organogenesis. The highest frequency of shoot bud regeneration was obtained from nodal callus (Table 2). The cultured explants gave rise to greenish compact callus by the end of 5th wk (Figs 2a & b). At the end of 10th wk, the maximum number of healthy shoot buds (7.2±0.84) were obtained in MS medium with 8 µ*M* BA and 2 µ*M* IBA from nodal explants (Fig. 2c). Multiple shoot buds were proliferated and elongated in the same medium. On the other hand, the leaf explant responded only for callus formation and no shoot formation was observed. However, the callus mediated regeneration of *J. curcas* was observed in other studies by culturing leaf and other explants on MS medium containing BA and IBA[3,15,21]. Thus, the results of present study along with earlier reports reveal that MS medium responds in varied ways depending upon explant type, growth regulator combination and the interactions *inter se*. Most probably, cytokinin at its high concentration stimulated development of meristems and at its optimal concentration promotes shoot proliferations,

Table 2—Effect of different plant growth regulators (PGRs) in combination on multiple shoot bud regeneration from nodal explants of *J. curcas* L. after 10 wk

MS medium with PGRs conc. (µm)			No. of shoots per explants (mean±SD)*
BA	IBA	2,4-D	
Control			1.4±0.55 ^{cde}
4.0	1.0	-	1.8±0.45 ^{def}
6.0	1.0	-	2.2±0.45 ^{fgh}
8.0	1.0	-	2.6±0.55 ^{ghi}
10.0	1.0	-	3.4±0.89 ^{j}
4.0	2.0	-	2.2±0.84 ^{fgh}
6.0	2.0	-	2.2±0.84 ^{fgh}
8.0	2.0	-	7.2±0.84 ^{l}
10.0	2.0	-	5.2±0.83 ^{k}
4.0	3.0	-	1.2±0.45 ^{cd}
6.0	3.0	-	1.8±0.45 ^{def}
8.0	3.0	-	2.0±0.71 ^{efg}
10.0	3.0	-	3.2±0.45 ^{ij}
4.0	4.0	-	0.4±0.55 ^{ab}
6.0	4.0	-	1.2±0.84 ^{cd}
8.0	4.0	-	2.8±0.45 ^{hij}
10.0	4.0	-	3.4±0.55 ^{j}
4.0	-	1.0	0.0±0.00 ^{a}
6.0	-	1.0	0.0±0.00 ^{a}
8.0	-	1.0	1.0±0.00 ^{bc}
10.0	-	1.0	1.8±0.45 ^{def}
4.0	-	2.0	0.0±0.00 ^{a}
6.0	-	2.0	0.0±0.00 ^{a}
8.0	-	2.0	1.8±0.45 ^{def}
10.0	-	2.0	2.4±0.55 ^{fgh}
4.0	-	4.0	0.0±0.00 ^{a}
6.0	-	4.0	0.0±0.00 ^{a}
8.0	-	4.0	0.0±0.00 ^{a}
10.0	-	4.0	0.0±0.00 ^{a}

*****Means within a column having the same letter are not statistically significant (p=0.05) according to Duncan's multiple range test (SPSS V 8.0.1)

and the inclusion of low concentration of auxin(s) along with cytokinin(s) triggers the rate of shoot proliferations.

Influence of additives was also studied in single and in various combinations after determining the optimum cytokinin and auxin levels for shoot regeneration from nodal explants. The effect of different additives was found to be insignificant. However, only one combination of additives (45 µ*M* adenine sulfate+15 µ*M* glutamine+10 µ*M* proline) gave the higher frequency (9.8±0.84) of multiple shoot bud regeneration (Fig. 2d) and elongation of shoots as compared to the control (MS medium+8 µ*M* BA+2 µ*M* IBA; 7.2±0.84) at the end of 4th wk (Table 3). Earlier, the positive influence of additives on the shoot proliferation rate and elongation of

Fig. 2 (a-h)—Plant regeneration in *J. curcas* L. through callus mediated organogenesis: Soft, greenish white calli developed from leaf explants after 5 wk (a), greenish, hard, compact calli developed from node explant after 5 wk on MS+8 µ*M* BA+2 µ*M* IBA (b), multiple shoot bud regeneration from the nodal callus after 10 wk (c), accelerated multiple shoot induction from nodal callus on MS+8 µ*M* BA+2 µ*M* IBA+45 µ*M* adenine sulfate+15 µ*M* glutamine+10 µ*M* proline (d), elongated shoot showing rooting through callus phase on MS+2 µ*M* NAA (e), elongated shoot showing rooting on hormone free MS media after overnight incubation on liquid MS+2 µ*M* IBA (f), roots showing abnormal origin of shoot on MS+2 µ*M* IBA (g), & a regenerated plantlets in plastic cups containing sand and soil mixture (h).

shoots in *J. curcas* has also been reported[17,31]. This enhanced rate of shoot regeneration might be attributed to the synergistic effect of additives with cytokinins on cell growth and shoot multiplication[32].

Rooting of Shoots

In present study, incubation of shoots in half-strength MS medium (2% sucrose) supplemented with 2µ*M* IBA for overnight followed by culturing on PGR free half strength MS medium provided better response for rhizogenesis both in term of rooting frequency as well as roots per shoot. The maximum number of roots (5.6±0.55) was observed in half-strength MS medium fortified with 2 µ*M* IBA (Fig. 2f), whereas maximum rooting frequency was observed in the combination of 2 µ*M* IBA and 2 µ*M* NAA (Table 4). In other studies also, IBA was reported as most effective auxin for *in vitro* rhizogenesis in *J. curcas*[3,13,17,33]. Moreover, the rooting was also reported on half-strength MS medium containing 26.85 µ*M* NAA[31]. However, in the present study, addition of NAA to the incubating

Table 3—Effect of different additives (adenine sulphate, glutamine and proline) on multiple shoot regeneration from node explants of *J. curcas* L. implanted on MS medium fortified with BA (8.0 µ*M*) and IBA (2.0 µ*M*) after 8 wk

Additives conc. (µ*M*)			No. of shoots per nodal explant (mean±SD) *
Adenine sulphate	Glutamine	Proline	
	Control		7.2±0.84[cd]
15.0	-	-	6.8±0.84 [bcd]
30.0	-	-	6.6±0.55 [bcd]
45.0	-	-	7.2±0.84[cd]
60.0	-	-	7.0±0.71 [bcd]
-	7.5	-	6.6±0.55 [bcd]
-	15.0	-	4.8±0.84 [a]
-	22.5	-	6.4±0.89 [abcd]
-	30.0	-	6.2±0.84 [abc]
-	-	5.0	6.0±0.71 [abc]
-	-	10.0	6.0±0.71[abc]
-	-	15.0	6.2±0.84[abc]
-	-	20.0	6.6±1.14 [bcd]
30.0	15.0	-	7.0±0.71 [bcd]
45.0	15.0	-	6.4±0.55 [abcd]
60.0	15.0	-	6.0±0.71 [abc]
30.0	15.0	10.0	6.8±1.48 [bcd]
30.0	15.0	15.0	7.6±0.89 [d]
30.0	15.0	20.0	6.8±0.84 [bcd]
45.0	15.0	10.0	9.8±0.84 [e]
45.0	15.0	15.0	6.0±1.00 [abc]
45.0	15.0	20.0	5.8±0.84 [ab]
60.0	15.0	10.0	6.6±0.55[bcd]
65.0	15.0	15.0	6.6±0.89[bcd]
60.0	15.0	20.0	6.6±0.55 [bcd]

*****Means within a column having the same letter are not statistically significant (p=0.05) according to Duncan's multiple range test (SPSS V 8.0.1)

medium promoted callus development at the distal end of the cultured shoot and after a prolonged period of culture rudimentary roots were developed (Fig. 2e). Those elongated shoots cultured in the rooting medium containing IBA without later replacing to PGR free half-strength MS medium gave rise to shoot from the developed roots (Fig. 2g). These results indicate that trhizogenesis in *J. curcas* requires auxin stimulation, while prolonged treatment with auxin promotes shoot development[34].

Acclimatization and Field Transfer

After 3 wk, the rooted plantlets were transferred into plastic pots containing autoclaved sand and soil (1:1) mixture and were maintained in the culture room for 2 wk (Fig. 2h). They were then transferred to shade and ultimately to field conditions. The survival rate of plantlets was 80±4% in the field. The regenerated plantlets did not show any variation in morphology or growth characteristics when compared with the donor plant.

Table 4—Influence of auxins on rooting of *in vitro*-derived shoots of *J. curcas* L. after 3 wk

MS(1/2) with auxin (µ*M*)		Rooting frequency (%)	No. of roots (mean±SD)*
IBA	NA A		
Control		-	0.0±0.00 [a]
0.4	-	53.33	1.0±0.45 [b]
1.0	-	46.66	1.8±0.45 [cd]
2.0	-	73.33	5.6±0.55 [f]
3.0	-	66.67	2.0±0.71[cd]
-	0.4	-	Callus; 0.0±0.00 [a]
-	1.0	-	Callus; 0.0±0.00 [a]
-	2.0	-	Callus; 0.0±0.00 [a]
-	3.0	-	Callus; 0.0±0.00 [a]
0.4	0.4	-	0.0±0.00 [a]
0.4	1.0	-	0.0±0.00 [a]
0.4	2.0	-	0.0±0.00 [a]
1.0	0.4	40.00	1.4±0.55 [bc]
1.0	1.0	53.33	1.4±0.55 [bc]
1.0	2.0	60.00	1.8±0.45 [cd]
2.0	0.4	60.00	2.4±0.55 [de]
2.0	1.0	66.67	3.0±0.71[e]
2.0	2.0	80.00	2.4±0.55[de]

*Means within a column having the same letter are not statistically significant (p=0.05) according to Duncan's Multiple range test (SPSS V 8.0.1)

Genetic Fidelity Assessment

The pre-existing variation in the cells of the donor explant and tissue culture induced genetic variation, particularly, in the callus phase, and could influence the genetic stability of regenerated plantlets. The usefulness of RAPD as a means of molecular analysis of *in vitro* regenerated plants has been well documented in several species[21,23-25]. In the present study, the genetic stability of the regenerated plants was screened by RAPD markers that could detect DNA sequence modifications at the primer annealing site of the genome. Forty primers (OPA 01-20 and OPB 01-20 series) were tested to analyze 11 regenerated plants as well as the donor parent. Among the 40 primers tested, 14 primers (Table 5) responded for genomic DNA amplification and produced 75 bands. All of them were highly reproducible with a mol wt between 160 to 2690 bp. All the responded primers revealed quite homogeneous and monomorphic banding pattern similar to the donor plant (Figs 3a-e: Table 5), which indicated lack of genetic variation in the plantlets in concordance with their morphological appearance.

Table 5—Details of RAPD analysis of *in vitro* regenerated plantlets using 14 decamer primers responded for amplification

Primer	Sequence (5′-3′)	No. of bands amplified	Range (bp)
OPA-01	CAGGCCCTTC	02	165-340
OPA-02	TGCCGAGCTG	05	450-2410
OPA-04	AATCGGGCTG	07	350-2050
OPA-08	GTGACGTAGG	07	290-2230
OPA-11	CAATCGCCGT	08	220-1735
OPA-13	CAGCACGCAC	11	270-1760
OPA-18	AGGTGACCGT	10	160-1680
OPA-20	GTTGCGTACC	02	250-800
OPB-02	TGATCCCTGG	03	750-1445
OPB-08	GTCCACACGG	06	240-1800
OPB-11	GTAGACCCGT	06	1035-2690
OPB-18	CCACAGCAGT	05	400-1900
OPB-19	ACCCCCGAAG	01	810
OPB-20	GGACCCTTAC	02	755-1020
Total		75	160-2690

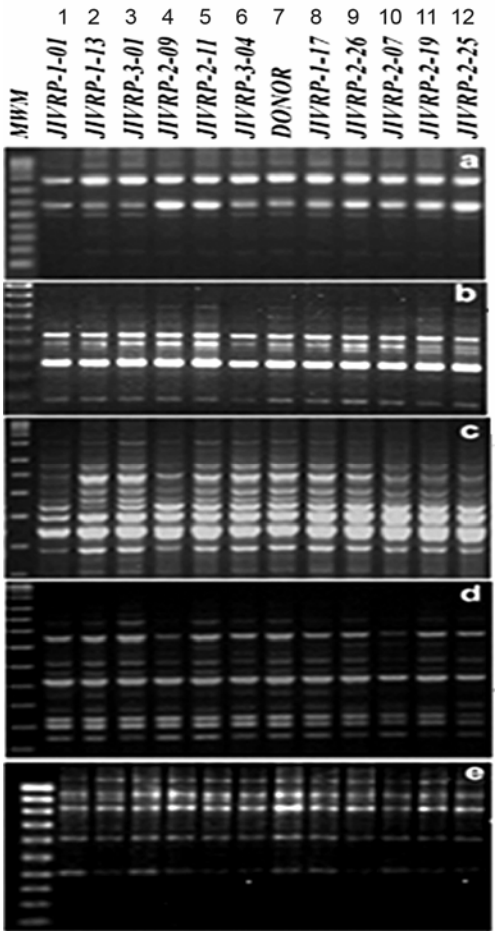

Fig. 3 (a-e)—Electrophoretic pattern of PCR amplified DNA fragments of the donor plant (L7) and eleven *in vitro* regenerants (L1-6 & L8-12) using primer OPA02 (a), OPA11 (b), OPA13 (c), OPA-18 (d), & OPB11 (e). The lane MWM represents mol wt marker containing 250 bp ladder.

In conclusion, the present efficient and reliable plant regeneration protocol can be potentially utilized for *ex-situ* conservation and mass propagation of *J. curcas* clones to meet the growing demand of energy plantations as well as need of herbal industry for therapeutics purpose. Future studies will focus on comparison between *in vitro* grown and field-grown plants in terms of their seed oil content and therapeutics activities.

Acknowledgement

Authors are grateful to the MITS Biodiesel Project (MBP), Government of Orissa, India for the financial assistance through the Departmental Research Scheme (MBP/MPTC/L-06/2006) to JP.

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