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In vitro regeneration and molecular characterization of *Jatropha curcas* plant

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Abstract

Background and objective: A simple, rapid, efficient, and reproducible protocol for callus induction and regeneration of plantlets from callus of *Jatropha curcas* plant was established.

Materials and methods: Randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analyses were used to determine the genetic variation between the regenerated, micropropagated, and mother plants.

Results: The highest callus induction percentage from leaf explant was recorded with MS medium containing 2.5 mg/l BA (*6-benzylaminopurine*) + 1.0 mg/l NAA (1-naphthaleneacetic acid). Leaf-derived callus was grown on medium containing 2.0 mg/l BA + 0.2 mg/l IBA (indole-3-butyric acid) for adventitious shoot regeneration. In addition, using five random RAPD primers with the tested samples produced 117 amplified products out of which 25 were polymorphic bands resulting in 21.37% polymorphism, whereas the five ISSR primers used yielded 116 scorable bands out of which 22 were polymorphic bands producing a polymorphism pecentage of 18.96.

Conclusion: An optimized protocol for large-scale production of *J. curcas* plants using plant biotechnology tools was achieved. RAPD and ISSR techniques would introduce an alternative system for large-scale production and establishment of genetically stable plants.

Keywords: *Jatropha curcas*, Callus induction, Regeneration, RAPD, ISSR

Background

Jatropha curcas L. belongs to family *Euphorbiaceae* and grows as a large shrub or small tree. The plant is found in tropical and subtropical areas, where it is used for reclaiming land and feedstuff; besides, the plant has different medicinal properties and many other uses. Due to the high oil content of *J. curcas* seeds (up to 60% oil) and low production cost, the plant attracted global attention for the development as a source for bio-fuel (Li et al. 2007).

Recent interest in promotion of *J. curcas* as a biodiesel crop demands genetic improvement of the crop for increased seed yield and oil content. Germplasm characterization is necessary to enhance germplasm management and utilization (Basha et al. 2009). A large number of polymorphic markers are required to measure genetic relationships and genetic diversity in a reliable manner. This limits the use of morphological characters and isozymes, which are few or lack adequate levels in *J. curcas* (Gupta et al. 2008). DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers (Soller and Beckmann 1983).

Over the last 15 years, polymerase chain reaction technology has led to the rise of two simple and quick techniques called randomly amplified polymorphic DNA (RAPD) (Li and Nelson 2002) and inter simple sequence repeats (ISSR) (Van der Nest et al. 2000) that were used in evaluation of *Jatropha* germplasm easily as their application does not need any prior sequence information (Gupta et al. 2008).

Jatropha is seen as a talented opportunity for the production of bio-fuel and is universally accepted as an energy crop. Potential improvement of *Jatropha* plant is

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currently a key target for scientists via agricultural practices and biotechnological approaches (Nitish and Muppala 2013). Conventional breeding has to be integrated with latest biotechnological approaches to introduce novel traits. The biotechnological means include propagation of selected genotypes using tissue culture techniques and further genetic engineering schemes. Biotechnological crop improvement thus appears as effective and alternative approaches. A particular emphasis on plant regeneration protocol from isolated plant cells or tissue is a prerequisite.

The present investigation has been organized to optimize a protocol for large-scale production of *J. curcas* plants using plant biotechnology techniques including callus induction and plant regeneration from callus tissues; the molecular analysis of the tissue culture raised plants in comparison with mother plants was investigated.

Materials and methods

Plant materials and culture conditions

Three months old *J. curcas* seedlings were kindly provided from the nursery of Ministry of Agriculture, Giza, Egypt. All media used were Murashige and Skoog (1962) (MS) medium with 30 g/l sucrose, and 7 g/l agar was added. The pH of medium was adjusted within a range of 5.6 to 5.8 by addition of 0.1 N potassium hydroxide (KOH) and 0.1 N hydrochloric acid (HCl). All media and all instruments (scalpel, forceps, scissors, and papers to work on) were sterilized by autoclaving at 121 °C and 15 psi for 20 min. All cultures were incubated under controlled conditions in the growth chamber. Temperature was 25 ± 2 °C, with photoperiod of 16 h light/8 dark, controlled automatically. Illumination intensity was ~ 2000 lux from cool fluorescent lamps (120-cm long).

Sterilization of *J. curcas* explants

Leaf explants were excised from *J. curcas* seedlings and rinsed in tap water, agitated in a disinfectant solution of 3% salvon (Carbendazim, BASF India Ltd.) for 30 min, and then soaked in 70% ethanol under aseptic conditions in a vertical air Laminar Flow Cabinet. Explants were immersed for 5 min in 0.1% mercuric chloride ($HgCl_2$), and then soaked for 10 min in 1.5% sodium hypochlorite solution (NaOCl). After each disinfection step, three rinses with sterile distilled water were applied. Sterilized leaf explants were excised to segments about 1 cm^2 for callus induction.

Callus induction

Three leaf explants were cultured on MS medium supplemented with different concentrations of BA and NAA. After 4 weeks of cultivation time taken for callus initiation (days), callus induction (%), fresh weight, dry weight (g), callus color, and morphology were recorded. The least significant difference (LSD) test at 0.05 level probabilities was used to compare mean values of all treatments.

Regeneration of plantlets from callus cultures

Leaf-derived calli of *J. curcas* were cultured on MS medium supplemented with different combinations of BA with 2,4-D or IBA for regeneration protocol. After 6 weeks of cultivation, regeneration percentage and number of regenerated adventitious shoots were recorded. Adventitious shoots raised from this experiment were applied to rooting experiment as done before (Rady et al. 2016).

Molecular analysis

Two PCR-based techniques, RAPD and ISSR, used for the evaluation of the genetic variance among tissue culture raised regenerated plants and micropropagated plants from our previous study (Rady et al. 2016) in comparison with mother plants. The use of these two types of molecular markers, which amplify different regions of the genome, allows better chances for identification of genetic variation between tested plants.

Genomic DNA isolation

Total genomic DNA was isolated from leaf explants according to Doyle and Doyle (1990).

RAPD-PCR analysis

A set of 5 random primers have been used for the evaluation of polymorphism percentage among the tested samples. RAPD-PCR amplification reactions were carried out according to the procedure described by Williams et al. (1990) with minor modifications. Reactions were occurred under PCR optimized conditions in 25 μl reaction volume containing 1× PCR buffer (Promega, USA), 1.5 mM $MgCl_2$, 2.0 mM deoxyribonucleoside triphosphates (dNTPs) (Promega, USA), 1.0 U *Taq* DNA polymerase (Bangalore Genei, India), 25 ng template DNA, and 1.0 μM primer from each of random primers (Operon) (Table 1), and finally, the volume completed up to 25 μl with double distilled water.

Amplification reaction was carried out using a Gene Amp PE applied Biosystem 9700 A (Perkin-Elmer, USA) thermocycler machine programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94 °C. Each cycle consisted of a denaturation step at 94 °C for 1.0 min, an annealing step at 36 °C for 1.0 min, and an elongation step at 72 °C for 1.5 min. The PCR products were incubated at 4 °C for further electrophoresis.

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) in 1.0× TBE buffer in the agarose gel electrophoresis system at 90 V for 1 h. DNA bands

Table 1 Sequence of primers used in randomly amplified polymorphic DNA (RAPD) analysis

Primer code	Primer sequence 5′ ---------- 3′
C12	GAGGCGTCGC
A6	TGGCCACCTG
AM7	CTTCGGCAGCATCTCTTCAT
AM8	CAGTGTGGAAGCCGATTATG
AM9	ATGTGTTGTCTGGCTTGGTA
AM1	CTCTCTCTCTCTCTCTG
AM2	GGATGGAATAGTCTC
AM3	GCATGGCAAGCTGCA
AM4	GAGAGAGAGAGAGAGAC
AM5	ACACACACACACACACC

were detected and photographed under UV light with gel documentation system. The size of amplification products was estimated using 3.0 kbp DNA ladder. The banding patters generated by RAPD analysis were compared to evaluate the genetic variance between samples. The amplified fragments were scored either as present (+) or absent (−). The genetic similarity coefficient (GS) between the tested samples was estimated according to Dice coefficient (Jaccard 1908). The similarity matrix was used in the cluster analysis which was used to organize the observed data into meaning for structures.

ISSR-PCR analysis

A set of 5 ISSR primers were used in the evaluation of polymorphism percentage among the tested samples. The amplification reaction (PCR) was performed in 25 μl reaction volume containing the following: 1× PCR buffer, 1.75 mg/l $MgCl_2$, 5 mM of each dNTPs, 1.0 U of *Taq* DNA polymerase, 25 ng of template DNA, and 40 μM of oligonucleotide ISSR primer (*Operon*) (Table 1), and finally, the volume completed up to 25 μl with double distilled water. PCR reaction was carried out in the thermocycler machine programmed to fulfill 35 cycles after an initial denaturation step for 5 min at 94 °C followed by ten touchdown cycles (94 °C/30 s, 65–55 °C/45 s, 72 °C/1 min). The PCR products were incubated at 4 °C. The amplification products were resolved by electrophoresis as mentioned before with RAPD analysis. The size of amplification products was estimated using the 3.0 kbp DNA ladder.

Results

Callus induction

The effect of different combinations of BA and NAA on callus initiation from leaf explants of *J. curcas* after 1 month of cultivation was investigated. The MS basal medium induces any callus tissues. The highest callus induction percentage was 97.5 on MS medium supplemented with 2.5 mg/l BA + 1.0 mg/l NAA (Fig. 1a). On the other hand, the lowest callus induction percentage (10) was observed with MS medium supplemented with 0.25 mg/l BA + 0.5 mg/l NAA (Fig. 1b). Moreover, the MS medium supplemented with 1.0 mg/l BA +0.5 mg/l NAA was the best medium giving the highest fresh weight from leaf explants (Fig. 1c), whereas the lowest fresh weight was obtained on MS medium supplemented with 0.25 mg/l BA + 0.5 mg/l NAA (Fig 1b).

Concerning callus color and morphology, a creamy white friable callus was showed in Fig 1a, c. Green friable (Fig. 1d) and creamy yellowish compact callus (Fig. 1e) was formed. In addition to creamy greenish compact callus (Fig. 1b), a deep green friable callus (Fig. 1f) was also noted.

Regeneration of plantlets from callus cultures

Effect of different concentrations of BA and 2,4-D or IBA on regeneration

From Table 2, it could be observed that the highest regeneration percentage was 83.33 recorded by MS medium supplemented with 2.0 mg/l BA + 0.2 mg/l IBA (Fig. 2a), followed by 66.67% that was reported by MS medium supplemented with 1.0 mg/l BA + 0.1 mg/l IBA (Fig. 2b), whereas MS medium supplemented with 0.5 mg/l BA + 1.0 mg/l 2,4-D gave the lowest regeneration percentage (21.67) (Fig 2c). Moreover, MS medium free of growth regulators did not show any response for regeneration of adventitious shoots (Fig 2d). Moreover, MS medium supplemented with 2.0 mg/l BA + 0.2 mg/l IBA gave the highest number of regenerated adventitious shoots (13.00), followed by 11.67 that was recorded with MS medium supplemented with 1.0 mg/l BA + 0.1 mg/l IBA. On the other hand, no regeneration of adventitious shoots was occurred when callus tissues was grown on medium without growth regulators.

Genetic variation based on RAPD analysis

Data in Table 3 demonstrated the results of RAPD analysis of six different samples of *J. curcas* plants mentioned as two samples from each of mother plant, in vitro propagated plant, and plants regenerated from callus. Out of the 10 random primers screened, only five primers produced clear reproducible bands. The five primers yielded 117 scorable bands (with an average of 23.4 bands per primer), including twenty-five polymorphic bands. The number of scorable bands from each primer varied from 16 to 38. The primer C12 in particular produced the highest number of strongly amplified individual fragments (38), whereas, primer AM9 produced the lowest number of amplicons (16). On the other hand, primer AM8 gave the highest percentage of polymorphism (38.88) while the lowest

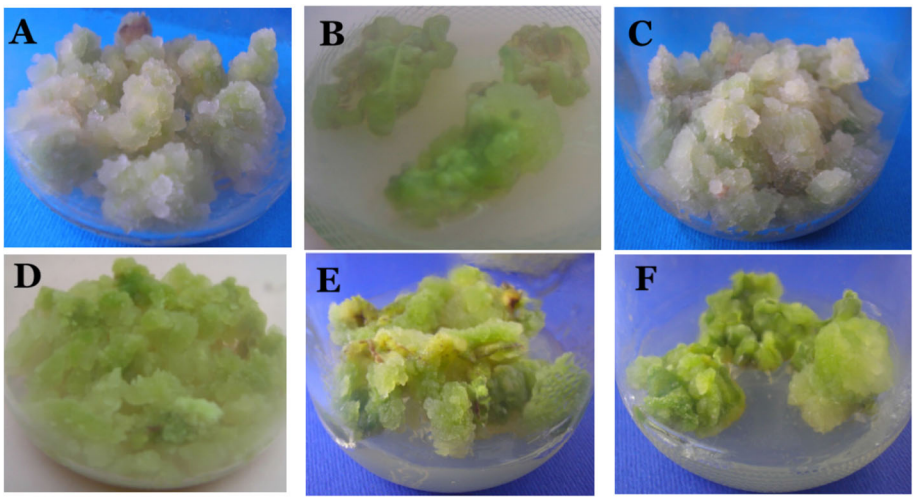

Fig. 1 Callus induction from leaf explant on MS medium containing:2.5 mg/l BA + 1.0 mg/l NAA (**a**), 0.25 mg/l BA + 0.5 mg/l NAA (**b**), 1.0 mg/l BA + 0.5 mg/l NAA (**c**), green friable callus grown on MS + 2.5 mg/l BA + 0.5 mg/l NAA (**d**), creamy yellowish compact callus grown on MS + 0.25, 0.5, or 1.0 mg/l BA in combination with 1.0 mg/l NAA (**e**), and a deep green friable callus grown on MS + 3.0 mg/l BA + 1.0 mg/l NAA (**f**) after 4 weeks of cultivation

percentage of polymorphism (14.29) was recorded with primer AM7. Using primer C12, 6 polymorphic bands out of 38 scorable bands were detected, representing polymorphism percentage of 15.79. Out of these six bands, three were absent with molecular weight 1134, 907, and 817 bp in regenerated plant number 2 (C2) lane 6 also, and three present polymorphic bands were detected with molecular weights of 881, 869, and 734 bp in C2 plant (lane 6), B2 plant (lane 4), and B1 plant (lane 3) respectively (Fig. 3a). And primer A6 produced a polymorphism percentage of 25, giving 6 polymorphic bands out of 24 amplification products. Two polymorphic bands of molecular weights of 900 and 468 bp in plant C2 (lane 6) were absent, whereas the other four polymorphic bands of molecular weights of 945, 658, and 525 bp in plant B2 (lane 4) and one at 451 bp in plants C2 (lane 6) were present (Fig. 3b). In addition, with primer AM7, 3 polymorphic bands out of 21 scorable bands were detected, representing a polymorphism percentage of 14.29. Three polymorphic bands with molecular weights of 726, 632, and 487 bp were absent in plant A1 (lane 1) (Fig. 3c), while primer AM8 produced 7 polymorphic bands out of 18 scorable bands, representing polymorphism a percentage of 38.88. Out of these 7 bands, 2 were absent with molecular weights of 865 and 374 bp in in vivo plants 1 (A1, lane 1) and in vivo plant 2 (A2, lane 2) respectively. Other five polymorphic bands were present with molecular weights of 575, 231, 224, 217, and 157 bp in in vivo plant 1 (A1, lane 1), in vitro plant 2 (B2, lane 4), regenerated plant 2 (C2, lane 6), regenerated plant 1 (C1, lane 5), and C1 (lane 5) respectively (Fig 3d). Finally, primer AM9 resulted in three polymorphic bands out of 16 scorable bands, representing a polymorphism percentage of 18.75. The three polymorphic bands were present with molecular weights of 843 bp presented in plant C2 (lane 6) and 620 and 412 bp in in vivo plant 1 (A1, lane 1) respectively (Fig. 3e).

Table 2 Effect of different concentrations of BA and 2,4-D or IBA on shoots regeneration from leaf-derived callus of *J. curcas* after 6 weeks of cultivation

Growth regulators (mg/l)			Regeneration (%)	No. of regenerated shoots
BA	2,4-D	IBA		
0.0	0.0	0.0	0.00	0.00
0.5	1.0	0.0	21.67	3.67
1.0	2.0	0.0	33.33	7.67
2.0	3.0	0.0	58.33	9.33
0.5	0.0	0.05	41.67	8.00
1.0	0.0	0.1	66.67	11.67
2.0	0.0	0.2	83.33	13.00
3.0	0.0	0.4	50.00	7.33

Unique markers as revealed by RAPD

Data tabulated in Table 4 showed the tested samples of *J. curcas* which characterized by unique positive and/or negative RAPD markers and the total number of markers identifying each sample. Different primers revealed various numbers of unique positive and/or negative markers across the six samples of tested plants. The total number of unique markers was 25, and 15 of them were representing unique positive marker and 10 represented the unique negative marker. The highest number

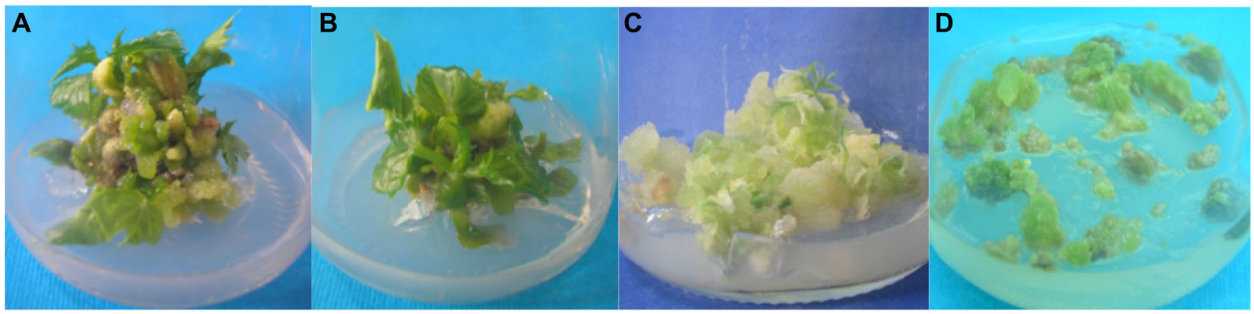

Fig. 2 Shoots regenerated from *J. curcas* leaf-derived callus cultured on MS medium with 2.0 mg/l BA + 0.2 mg/l IBA (**a**), 1.0 mg/l BA + 0.1 mg/l IBA (**b**), 0.5 mg/l BA + 1.0 mg/l 2,4-D (**c**), and control (**d**), after 6 weeks of cultivation

of unique markers (9) was exhibited with regenerated plant 2 (C2). C2 showed 9 unique markers, and 4 of them were unique positive and were scored at molecular sizes of 881, 451, 224, and 843 bp with primers C12, A6, AM8 and AM9 respectively. The rest (5) were unique negative markers that were scored with primer C12 at molecular sizes of 1134, 907, and 817 bp and primer A6 at molecular sizes of 900 and 468 bp. In addition, with in vivo plant 1 (A1), 3 unique positive markers out of 7 unique markers were reported. One of them was scored at 575 bp with primer AM8 and 2 were scored at 620 and 412 bp with primer AM9, whereas for the 4 unique negative markers, 3 of them were scored at 726, 632, and 487 bp with primer AM7 and one were scored at molecular size of 865 bp with primer AM8. And with in vitro plant 2 (B2), it exhibited five unique markers; all of them were unique negative markers. One of them was scored with primer C12 at 869 bp, 3 of them were scored at 945, 658, and 525 bp with primer A6, and one was scored at 231 bp with primer AM8. Moreover, the lowest number of unique markers was the one which appeared with in vivo plant 2 (A2) as a unique negative marker scored at 374 bp with primer AM8; also, one unique marker was exhibited in in vitro plant 1 (B1) as a unique positive marker scored at 734 bp with primer C12. From the obtained data, it could be observed that regenerated plant 1 (C1) exhibited 2 unique markers with primer AM8 scored as unique positive markers at molecular sizes 217 and 157 bp.

Cluster analysis of *J. curcas* tested samples

The dendrogram constructed from UPGMA cluster analysis of the Dice similarity coefficients calculated from RAPD data is shown in Fig. 4. The dendrogram based on genetic similarities separated the six samples of *J. curcas* into two main groups.

The regenerated plant 2 (C2) was grouped in the first cluster alone, and all other samples were grouped in the second cluster, which was separated into two sub-clusters; the first sub-cluster included in vivo plant 1 (A1) and the second included the other 4 samples (A1, B1, B2, and C1). The 4 samples were classified into two sub-clusters, the first included regenerated plant 1 (C1) and the second included the other 3 samples sub-cluster. This sub-cluster divided into two sub-clusters; the first included in vitro plant 2 (B2) and the second included in vivo plant 2 (A2) and in vitro plant 1 (B1).

Table 3 RAPD-PCR amplification products of DNA extracted from different samples of *J. curcas* plants using different five random primers

Primer code	Primer sequence 5′ ---------- 3′	Total number of Scorable bands	Number of monomorphic bands	Number of polymorphic bands	Polymorphism (%)
C12	GAGGCGTCGC	38	18	6	15.79
A6	TGGCCACCTG	24	6	6	25.00
AM7	CTTCGGCAGC--ATCTCTTCAT	21	6	3	14.29
AM8	CAGTGTGGAA--GCCGATTATG	18	0	7	38.88
AM9	ATGTGTTGTC--TGGCTTGGTA	16	6	3	18.75
Overall totals	----------	**117**	**36**	**25**	**21.37**

Polymorphism% = (number of polymorphic bands/total number of scorable bands) × 100

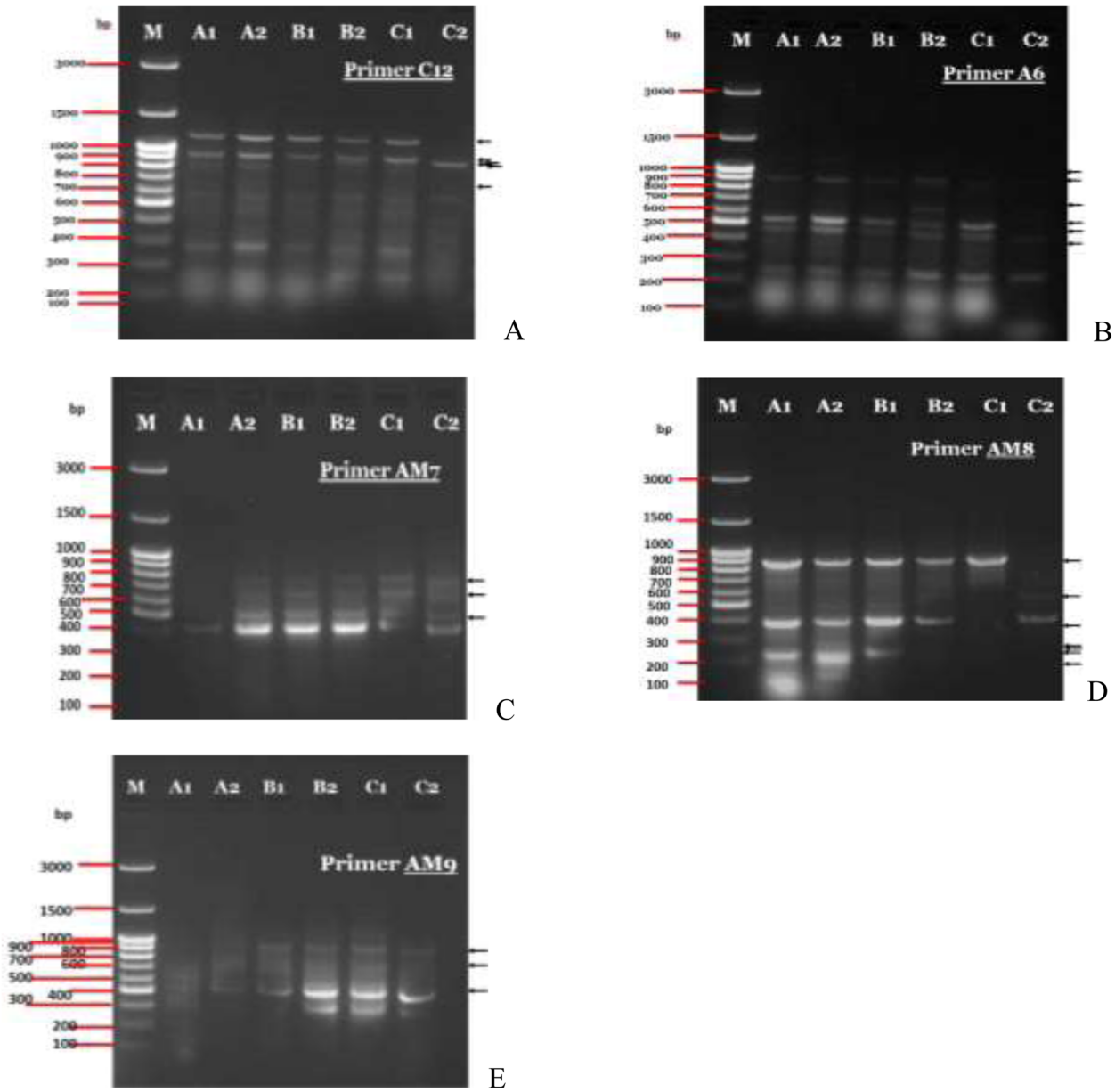

Fig. 3 Agarose gel electrophoresis of randomly primed DNA amplification (RAPD) fragments. Lanes A1 and A2 refer to mother plants, lanes B1 and B2 refer to in vitro propagated plants, lanes C1 and C2 refer to in vitro plants regenerated from callus, and lane M indicates molecular weight DNA ladder given in bp (100–3000). Polymorphic DNA fragments are identified by an arrowhead

Inter simple sequence repeat (ISSR) analysis

Data in Table 5 demonstrated the results of ISSR analysis of three different samples of *J. curcas* plants. Out of ten ISSR primers screened, only five primers produced clear reproducible bands. The five primers yielded 116 scorable bands (with an average of 23.2 bands per primer), including 22 polymorphic bands. The number of scorable bands from each primer varied from 14 to 30, while Fig. 5 showed the distribution and band sizes of produced by each primer. The number of bands varied from 14 (primer AM2) to 30 (primer AM1), and sizes ranged from 185 to 1571 bp. The average numbers of polymorphic bands per primer was 4.4. The percentage of polymorphism ranged from 10 recorded with primer AM1 to 31.82 recorded with AM5 primer with an average polymorphism of 18.96% across all tested samples.

Using primer AM1, 3 polymorphic bands out of 30 scorable bands were detected, representing polymorphism percentage of 10. The three bands were absent at

Table 4 Unique markers based on RAPD for different samples of *J. curcas*

Code (lane)	Sample	Primer	UPM (bp)	UNM (bp)	Total for	
					Primer	Sample
A1 (1)	In vivo (1)	AM7	–	726 632 487	3	7
		AM8	575	865	2	
		AM9	620 412	–	2	
A2 (2)	In vivo (2)	AM8	–	374	1	1
B1 (3)	In vitro (1)	C12	734	–	1	1
B2 (4)	In vitro (2)	C12	869	–	1	5
		A6	945 658 525	–	3	
		AM8	231	–	1	
C1 (5)	Regenerated (1)	AM8	217 157	–	2	2
C2 (6)	Regenerated (2)	C12	881	1134 907 817	4	9
		A6	451	900, 468	3	
		AM8	224	–	1	
		AM9	843	–	1	
Total			15	10		25

UPM unique positive marker, *UNM* unique negative marker

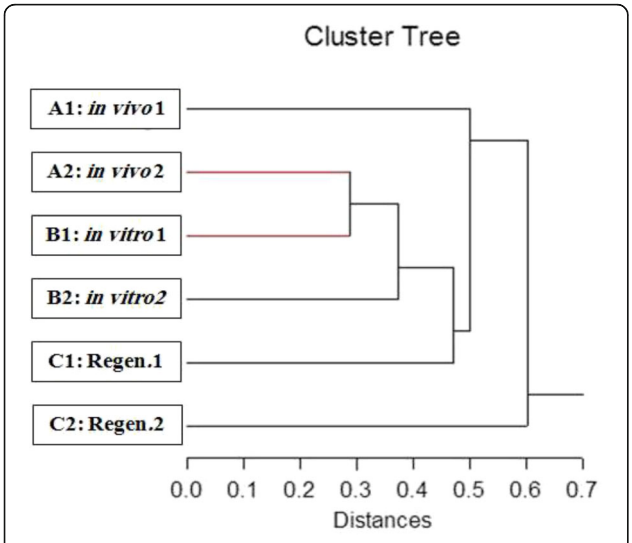

Fig. 4 Dendrogram for six samples of *J. curcas* plant (2 samples of each of in vivo plants, in vitro plants, and plants regenerated from callus) generated UPGMA cluster analysis of the similarity values

molecular size of 900 bp with micropropagated plant, lane (2), and 800 and 205 bp with mother plant, lane (1) (Fig. 5a). With regard to the results, it could be observed that primer AM2 produced a polymorphism percentage of 28.57, recording 4 polymorphic bands out of 14 amplification products (Fig. 5b). The 4 bands were absent bands at molecular sizes of 1456, 812, and 506 bp in the regenerated plant (lane 3) and at 450 bp in the mother plant (lane 1). In addition, with primer AM3, 3 polymorphic bands out of 23 scorable bands were detected representing polymorphism percentage of 13.04 (Fig. 5c). Two of them were absent in mother plant A1 (lane 1) with molecular weights of 1436 and 1014 bp, and one was present at the molecular size of 681 bp in the micropropagated plant B1 (lane 2). Moreover, primer AM4 produced 5 polymorphic bands out of 27 scorable bands, representing polymorphism percentage of 18.52 (Fig. 5d). Out of these 5 bands, 3 were absent in micropropagated plant B1 (lane 2) at molecular sizes of 1571, 808, and 318 bp; one was absent in mother plant A1 (lane 1) at molecular size of 919 bp. Moreover, one band was present in mother plant A1 (lane 1) at a molecular size of 716 bp. Using primer AM5 resulted in 7 polymorphic bands out of 22 scorable bands, representing a polymorphism percentage of 31.82. Out of them, 4 were absent in regenerated plant C1 (lane 3) at molecular sizes of 1571, 874, 765, and 438. Moreover, two bands were absent in the micropropagated plant B1 (lane 2) at molecular sizes of 1151 and 1000 bp (Fig. 5). Finally, one band was present at molecular size of 733 bp in mother plant A1 (lane 1) (Fig. 5e).

Discussion

The best treatment for callus induction from leaf explant of *J. curcas* were MS medium supplemented with 2.5 mg/l BA and 1.0 mg/l NAA. In this respect, Rajore and Batra (2007) induced callus from leaf explants of *J. curcas* by culturing it on MS media supplemented with 1.0 mg/l NAA and 5.0 mg/l BAP. Moreover; Kumar et al. (2008) cultured leaf explant of *J. curcas* on MS medium supplemented with 5 mg/l BAP and 1 mg/l NAA obtaining a friable callus that used for evaluation of dry and fresh mass of the grown callus. Yan Bi et al. (2009) showed that the best medium for callus induction from cotyledon of *J. curcas* plant was MS medium supplemented with 2.0 mg/l BA in combination with 2.0 mg/l NAA. Also, Mohanalakshmi et al. (2009) obtained callus when they subcultured different explants of *J. curcas* on MS supplemented with 1.5 mg/l BA only, while Varshney and Johnson (2010) showed that MS medium supplemented with 0.5 mg/l IBA and 1.0 mg/l BA was the best medium for callus induction from immature embryos of *J. curcas* giving a response of morphogenic callus induction (85.7%). Moreover, Meng Ji et al. (2010)

Table 5 ISSR-PCR amplification products of DNA extracted from different samples of *J. curcas* plants using different five ISSR primers

Primer code	Primer sequence 5′ ---------- 3′	Total number of scorable bands	Number of monomorphic bands	Number of polymorphic bands	Polymorphism (%)
AM1	CTCTCTCTCTCTCTCTG	30	24	3	10
AM2	GGATGGAATAGTCTC	14	6	4	28.57
AM3	GCATGGCAAGCTGCA	23	18	3	13.04
AM4	GAGAGAGAGAGAGAGAC	27	18	5	18.52
AM5	ACACACACACACACACC	22	9	7	31.82
Overall totals	----	116	75	22	18.96

Polymorphism % = (number of polymorphic bands/total number of scorable bands) × 100

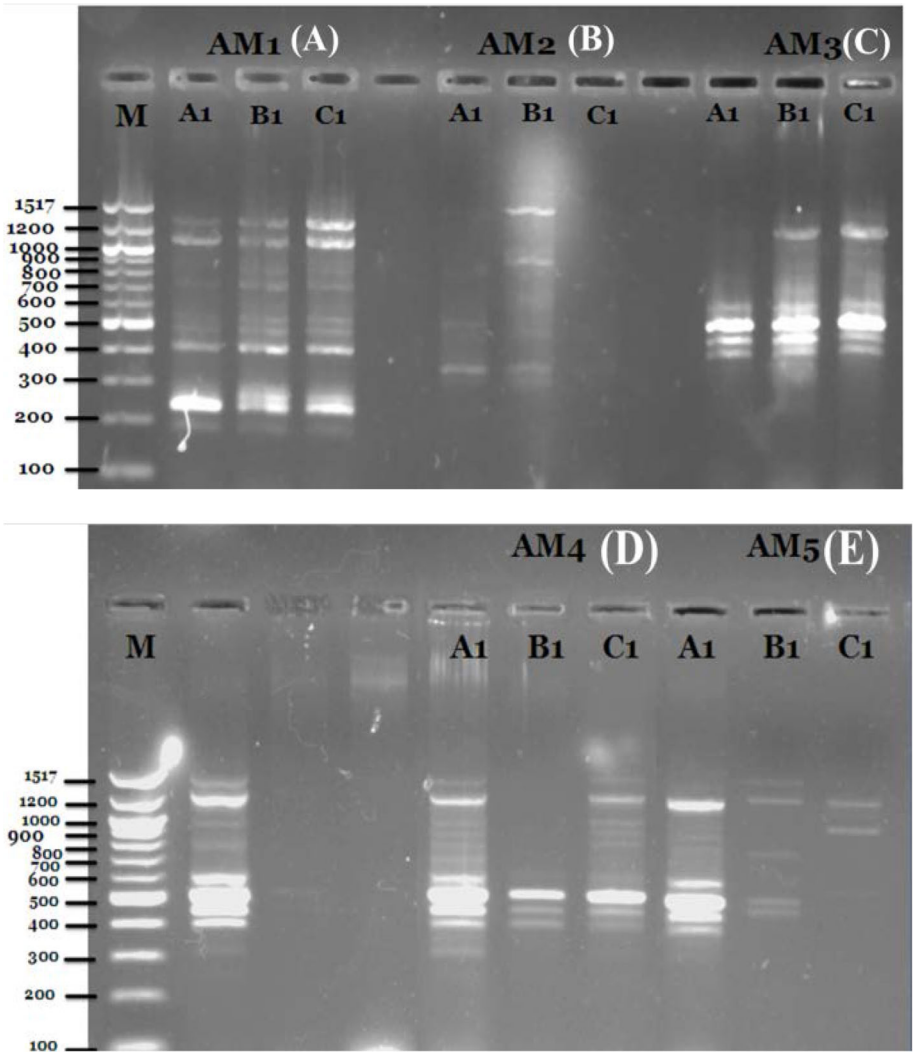

Fig. 5 Agarose gel electrophoresis of inter simple sequence repeat (ISSR) fragments. Lane 1 (A1) refers to mother plants, lane 2 (B1) refers to in vitro propagated plants, lane 3 (C1) refers to plants regenerated from callus, and lane M indicates molecular weight DNA ladder given in bp (100–1517). Polymorphic DNA fragments are identified by an arrowhead

showed that 0.5 mg/l BA + 0.1 mg/l 2,4-D was the best medium for callus induction rate (96.7%) from *J. curcas*. Misra et al. (2010) reported that the optimum treatment for callus induction in leaf segments of *J. curcas* plants was 0.5 mg/l BA with 1.0 mg/l each of 2,4-D and IAA obtaining both types of calli (green and compact; yellow and friable), and they were employed for shoot organogenesis. Also, Ranwah et al. (2009) observed a green callus when they incubated leaf discs of *J. curcas* on MS medium supplemented with 10 mg/l BAP in combination with 5 mg/l IBA. Moreover, by sub-culturing on the same medium, they found that the quantity of callus was increased.

Different authors reported the regeneration of plantlets from different explants of *J. curcas* when explants were cultured on different media. In this connection, Sardana et al. (2000) obtained embryogenic callus from leaf explants of *J. curcas*. They noticed the formation of plantlets when the embryogenic callus was cultured on MS medium supplemented with 3.0 mg/l GA_3 in combination with 1.0 mg/l IAA. Also, Sharma et al. (2006) obtained shoot buds from callus tissues when grown on MS medium supplemented with 0.5 mg/l NAA and 0.06 mg/l zeatin. Moreover, Rajore and Batra (2007) used green compact nodules of callus tissues and found that MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l IBA was the best combination for shoot bud induction and elongation. In addition, Thepsamran et al. (2008) reported that MS medium supplemented with 1.0 mg/l BA in combination with 0.5 mg/l IBA was the best medium for regeneration of adventitious shoots from callus induced from petioles of leaves of *J. curcas*. In addition, our results are in agreement with Verma et al. (2008). They induced callus from petiole explants of *J.* curcas and found that MS medium supplemented with 0.25 mg/l IBA in combination with 2.0 mg/l BAP was the best medium for shoot regeneration (75%) from callus tissues. Also, regenerated adventitious shoots of *J. curcas* was obtained from induced callus tissues (Mohanalakshmi et al. 2009). They reported that MS medium supplemented with 1.0 mg/l BA + 1.5 mg/l NAA was the best medium for regeneration of adventitious shoots. Recently, BoBin et al. (2010) found that the best combination for callus induction and induction of adventitious shoot buds (75.8%) was MS medium supplemented with 5.0 mg/l BA and 0.5 mg/l IBA.

Regarding RAPD analysis, the present results were in common with those obtained by many researchers. In this respect, Yadav and Shukla (2010) assessed the genetic diversity among different 20 genotypes of *J. curcas* plants using 20 random RAPD primers showing 158 reproducible bands. Each of LC-77, LC-84, and LC-96 primers produced about 4 bands, whereas LC-89 primer produced about 22 bands with an average of 7.9 bands per primer. Each primer showed 100% of polymorphism. Moreover, Subramanyam et al. (2010) assessed the genetic diversity and pedigree of 10 accessions of *J. curcas*, collected from different Indian regions. Ten selected primers produced 125 bands from which 76 were polymorphic bands. Each primer produced an average 12.5 bands, of which 7.6 were polymorphic bands. Also, Ikbal et al. (2010) used RAPD technique utilizing 50 random primers to assess the genetic diversity among different accessions of *J. curcas* collected from different eco-geographical Indian regions. From which 44 primers produced 308 polymorphic bands out of 328. The polymorphism was scored and used in band sharing analysis to identify genetic relationship.

Sharma et al. (2011) studied the genetic stability of in vitro *J. curcas* plantlets regenerated from axillary buds obtained from selected high-yielding genotypes using RAPD analysis. Out of 52 screened RAPD primers, 21 primers gave clear reproducible bands. In the micropropagated plantlets, 4 bands out of 177 scorable bands were polymorphic within the 2nd sub-culture, whereas no polymorphisms were detected in the 8th and 16th sub-cultures. They concluded that axillary shoot proliferation can safely be used as an efficient micropropagation method for mass propagation of *J. curcas* giving a high genetic stability for micropropagated plantlets of *J. curcas*. Also, Mastan et al. (2012) used the RAPD technique to assess the genetic diversity among elite germplasms of *J. curcas*. They found that, out of 250 amplicons, 141 found to be polymorphic using 26 RAPD primers. Polymorphism percentage among the selected germplasms using RAPD was found to be 56.43. Recently, Gautam Murty et al. (2013) evaluated the genetic diversity of 19 *Jatropha* accessions using RAPD in combination with ISSR and DAMD techniques. RAPD analysis produced a polymorphism percentage of 96.67.

Data of unique markers revealed by RAPD analysis of the tested *J. curcas* samples showed that primer AM8 exhibited the highest number of unique markers (7) mentioned as the following: two with in vivo plant 1 (A1), one with in vivo plant 2 (A2), one with in vitro plant 2 (B2), 2 with regenerated plant 1 (C1), and 1 with regenerated plant 2 (C2). The lowest number of unique markers (3) was exhibited with primers AM7 and AM9. All unique markers with AM7 appeared with in vivo plant 1 (A1), while 2 unique markers appeared with in vivo plant 1 (A1) and one with regenerated plant 2 (C2) for primer AM9. Six unique markers appeared with each of primers C12 and A6. Many authors reported that RAPD-PCR markers gave adequate distinctions among all the tested cultivars of different crops. In this respect, Adawy and El-Sherbieny (1999) studied genotype identification and genetic distance among maize inbred lines using RAPD markers. They identified 28

unique markers distributed among maize inbred lines from three populations. Abdel-Tawab et al. (2001) found some unique bands which could be used as a DNA marker for cultivar identification in sweet sorghum. Also, El-Khishin et al. (2003) surveyed 20 maize inbred lines using 40 RAPD markers, and 21 of them gave unique markers which identified individual lines from each other.

Many workers used RAPD as a tool for determining the extent of genetic diversity among different crops and for allocating genotypes into different groups. In this connection, Ram et al. (2008) studied the genetic diversity of 12 species of *Jatropha* and reported that Jaccard's similarity coefficient indicated a high level of genetic variance among studied genotypes as it varied from 0.00 to 0.85. Three main clusters was indicated by UPGMA cluster analysis, one including all accessions of *J. curcas*, while the second included six species, and the third cluster contained 2 species, indicating its higher genetic distinctness from other species.

Jubera et al. (2009) studied the genetic variation in seven genotypes of *J. curcas* using RAPD analysis. They found that Jaccard's similarity coefficient showed a high genetic similarity among studied genotypes as it ranged from 81.8 to 100. Dendrogram analysis showed two main clusters of the seven studied genotypes. Also, Subramanyam et al. (2009) used RAPD technique to identify the genetic diversity in 40 accessions of *J. curcas*. They reported that Jaccard's similarity coefficient varied from 0.00 to 1.00, indicative of high levels of genetic variation among the genotypes studied, whereas cluster analysis of data using UPGMA algorithm placed the 40 accessions into 2 main clusters, with cluster II divided into six subclusters. Sarkar et al. (2010) used the RAPD technique to identify the polymorphism in *J. curcas* plants raised in vitro from leaf explants. The dendrogram analysis showed three groups, A, B, and C. One hundred percent genetic similarity was detected within group A samples, whereas a higher genetic variance was detected between samples from groups B and C as compared to samples from A and B as well as A and C. Moreover, Ikbal et al. (2010) studied the genetic diversity among different accessions of *J. curcas*. Cluster analysis based on Jaccard's similarity coefficient using UPGMA grouped all the 40 genotypes into two major groups at a similarity coefficient of 0.54. Similarity indices ranged from 0.44 to 0.92 with an average of 0.73, indicating a moderate to high genetic variability among the genotypes. Recently, Alkimim et al. (2013) estimated the genetic diversity among 46 accessions of *J. curcas* using Nei and Li's similarity coefficient based on 69 RAPD primers and ISSR technique. The genetic distance between accessions ranged from 0.13 to 0.76. The most divergent genotypes were 86, 71, and 83. A dendrogram based on UPGMA of the joint data matrix presented only two phylogenetic groups, one of which contained only three individuals; the remaining group included 95.6% of the analyzed genotypes.

The results of this study could identify polymorphic ISSR markers that could distinguish between mother plant and tissue culture-raised plants. The current study with ISSR markers has revealed a low level of polymorphism between the tested samples. In this respect, Cai et al. (2010) studied the genetic diversity of Chinese *Jatropha* germplasm using ISSR markers. They found 127 polymorphic bands out of 169 amplified bands producing a polymorphism pecentage of 75.15. Also, Grativol et al. (2011) studied the genetic diversity of 332 Brazilian *J. curcas* accessions using ISSR markers. Seven ISSR primers produced 21,253 amplified bands out of which 19472 were polymorphic producing 91% polymorphism. In addition, Arolu et al. (2012) used 10 ISSR primers for characterization of 48 *J. curcas* Malaysian accessions. They reported that the polymorphism pecentage ranged from 90.75 to 100. Camellia et al. (2012) used 8 ISSR primers for evaluation of genetic relationship among 16 accessions of *J. curcas*. They found that 25 polymorphic bands out of 63 amplified bands resulting in 40% polymorphism. Moreover, Khurana-Kaul et al. (2012) used 25 ISSR primers to assess the genetic relationships of 29 *J. curcas* accessions and found that the polymorphism percentage was 59.8. Recently, Biabani et al. (2013) employed 10 ISSR primers to assess the genetic diversity among 6 populations of *Jatropha* from different Asian countries. One hundred forty-three polymorphic bands were produced, and the polymorphism pecentage ranged between 46.2 and 60.8 between the different genotypes. Generally, most workers obtained high percentage of polymorphism, and this may be because of the analysis of different species and genotypes of *Jatropha* from different regions. But in this study, low polymorphism percentage was detected in comparison to other workers. This may be due to the analysis of samples in the same genotypes of *J. curcas* (mother, micropropagated, and regenerated) plants.

Conclusion

An optimized protocol for large-scale production of *J. curcas* plants using plant biotechnology tools was achieved. RAPD and ISSR techniques would introduce an alternative system for large-scale production and establishment of genetically stable plants.

Abbreviations

RAPD: Randomly amplified polymorphic DNA; ISSR: Inter simple sequence repeat; NAA: 1-Naphthaleneacetic acid; BA: 6-Benzylaminopurine; IBA: Indole-3-butyric acid

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