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Evaluation of Somaclonal Variation in Callus Cultures of *Jatropha curcas* Maintained on Different Hormonal Combinations Using RAPD Markers

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Abstract: Random genetic changes generated during *in vitro* culture are not desirable for plant micropropagation and genetic transformation. RAPD markers were used to detect the variation in leaf disc callus cultures of *Jatropha curcas*, maintained in Murashige and Skoog (MS) medium with different auxin and cytokinin combinations. In total 41 scorable bands were produced with 11 primers. Out of 41 bands, 37 were polymorphic (91.12%). The average number of polymorphic bands was 3.36 per primer. The highest similarity (0.82) with mother plant was seen in callus maintained on MS with hormonal combination Indole butyric acid - 0.4mg/l+ N6-benzyladenine purine - 4.0 mg/l. The callus grown on MS with hormonal combinations IBA- 0.4mg/l+ BAP- 2.0mg/l, IBA- 0.4mg/l+ BAP- 2.5mg/l and IBA- 0.6 mg/l+ BAP- 2.0 mg/l also showed similarity with the mother plant. Callus maintained on MS with hormonal combination IBA- 0.2mg/l+ BAP- 2.0 mg/l was found to show least similarity (0.53) with mother plant.

Key words: BAP · IBA · *Jatropha curcas* · RAPD · Somaclonal variation

Abbreviations:

BAP	6-Benzylaminopurine
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
RAPD	Random amplified polymorphic DNA

INTRODUCTION

Jatropha curcas L. (tropical physic nut) is a perennial multipurpose shrub belonging to the family Euphorbiaceae [1]. It is a popular plant for its value in the biofuel, cosmetics and biopesticides industries [2]. Tissue culture techniques offer rapid and continuous supply of the planting material circumventing the problems associated with large scale multiplication of *J. curcas* [3]. Evaluation of tissue culture propagated plants of *J. curcas* revealed that these are better than seed propagated plants in terms of yield and yield related traits [4].

The growth regulators used in *in vitro* culture techniques can contribute to somaclonal variation. Generation of callus cultures involves cell adaptation to the conditions of *in vitro* culturing and requires changes that are beyond the reaction norm of the genome. This results in destabilizing selection and an increase in genetic variation during early stages of culture [5]. In recent years considerable amount of evidence has accumulated indicating that all classes of plant hormones can cause selective changes in the levels of specific mRNAs [6-9]. DNA sequence variation can be identified using RAPD (Random amplified polymorphic DNA) technique, as RAPD markers are not typically influenced by environmental conditions or the developmental stage [10, 11]. RAPD polymorphism results from either a nucleotide base change that alter the primer binding site, or from an insertion or deletion within the amplified region. Polymorphism usually results in the presence or absence of amplification products from a single locus [12].

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RAPD markers may reveal a differential effect of cytokinin/auxin on genomic DNA of callus tissues and help identify auxin and cytokinin combinations that produce more (or less) genetic variation in *Jatropha curcus* callus tissues.

In the present study, we report the use of RAPD markers, for the assessment of genetic variation of callus grown in different hormonal combinations in comparison with the mother plant.

MATERIALS AND METHODS

Mother plants of *Jatropha curcas* were a kind gift from Enhanced Biofuels & Technologies India Private Limited, Coimbatore. The leaf explants (2nd and 3rd leaves) were surface sterilized with Tween 20 and 0.1% (w/v) Mercuric chloride. After sterilization the explants were trimmed into leaf discs of ~0.3mm in diameter and placed with the abaxial side in contact with the medium. For culturing the leaf discs, Murashige and Skoog [13] basal medium supplemented with 3% (w/v) sucrose was used. It was fortified with different combinations (S1- S8) of auxin (IBA- Indole butyric acid) and cytokinin (BAP-N6-benzyladenine purine) which varies from 0 - 0.6mg/l and 1.0mg/l- 5.0mg/l respectively as detailed in Table 1. Agar at 0.8% (w/v) (Hi media, India) was used as solidifying agent. All experiments were carried out in culture tubes (150×25 mm) containing 20 ml of culture medium. The pH of media was adjusted to 5.7±0.1 prior to autoclaving at 121°C at 1.1kg sq.cm for 15 minutes. Cultures were incubated under 16h/8h light/dark cycles at 23±2°C.

Genomic DNA for PCR was isolated from calli grown in MS media supplemented with 8 different hormonal combinations (S1-S8) and from the leaves of the mother plants (S9) by Doyle and Doyle [14] method using CTAB (Cetyltrimethylammonium bromide).

The yield of DNA extracted was measured using a UV spectrophotometer (UV-1601 Shimadzu, Kyoto, Japan) at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity was also determined by running the samples 1.0% Agarose gel, based on the intensities of band when compared with 250 bp DNA marker from Chromous Biotech (Bangalore, India).

DNA extracted from *Jatropha curcas* callus induced in different hormonal combinations was used for RAPD analysis with 38 oligonucleotide primers from Operon Technologies Inc. (Almeda CA, USA). Reactions without DNA were used as negative controls. Each 20µl reaction volume contained about 50 ng of template DNA, 1X PCR Buffer (10mM Tris HCl pH 8.3; 50mM KCl), 0.2 mM dNTP Mix, 0.5µM of single primer (Operon Technologies, Inc., Almeda CA, USA), 1 U of *Taq* DNA polymerase (Sigma Aldrich, USA). Different concentrations (1.5- 3.5 mM) of Magnesium chloride ($MgCl_2$) were tried to optimize the reaction.

The thermocycler (Biorad MJ Mini thermal cycler, USA) was programmed for an initial denaturation step of 5 minute at 94°C, denaturation at 92°C for 1 minute, annealing for 1 minute at 35°C, extension was carried out at 72°C for 2 minutes and final extension at 72°C for 10 minutes and a hold temperature of 4°C at the end. PCR products were electrophoresed on 2% (w/v) agarose gels, in 1X TAE Buffer at 120 V for 2hrs and then stained with Ethidium bromide (0.5µg/ml). Gels with amplicons were visualized and photographed in Gel Documentation system (Syn gene). Each RAPD product was assumed to represent a single locus and data were scored for presence (1) and absence (0) of bands. Index of genetic variation was calculated and dendrograms were constructed through UPGMA method using computer software POPGENE (ver. 1.32) of Yeh *et al*. [15].

Table 1: Callus proliferation in MS media with different hormonal combinations from *Jatropha curcas*

Samples	Hormonal combination on IBA+ BAP (mg/l)	Type of callus
S1	0.0 + 2.0	Less friable callus formation with expansion of the leaf disc
S2	0.2 + 2.0	White to light green soft callus
S3	0.3 + 1.0	Light to pale green, compact, callus
S4	0.3 +.2.5	Dark green, compact, callus
S5	0.4 + 2.0	Dark green, compact, hard callus
S6	0.4 + 2.5	Light to dark green, compact, callus
S7	0.4 + 4.0	Light to dark green compact callus
S8	0.6 + 2.0	White to light green hard, compact callus

RESULTS

Callus tissue was initiated from leaf explants along the cut portions after two days of inoculation. A wide range of variation in frequency of callus formation and nature of callus was observed depending on the concentration and combination of hormones used. At lower concentration of IBA, white to light green colored callus was formed, whereas at higher concentrations dark green callus was induced. MS media supplemented only with BAP (2.0 mg/l), showed very little callus formation (Table 1). Higher frequency of callus induction was observed in MS medium supplemented with IBA- 0.4 mg/l and BAP- 2.5 mg/l. Compact callus was observed with light to green color (Fig. 1).

The DNA extracted from the callus samples S1-S8 and mother plant (S9) was of good quality with absorbance at 260/280 nm between 1.741-1.946. Of 38 decamer primers used for the RAPD analysis, 11 primers showed consistent band patterns. In total, 41 scorable bands were observed with the 11 primers (Table 2).

The total number of amplicons produced per primer varied from 2 for OPA05, OPD07 (Fig. 2a and d) and OPM12 (Fig. 2k) to as many as 7 bands for OPE01 (Fig. 2f) and OPK12 (Fig. 2i). The average number of bands per primer was 3.72. Out of 41 bands, 37 were polymorphic (91.12%). The average number of polymorphic RAPD bands was 3.36 per primer. Polymerase chain reaction with RAPD primers OPA11, OPC08, OPE01and OPH-18 showed one monomorphic band in all samples (S1-S8 and in mother plant genomic DNA).

Considering the dendrogram (Fig. 3) constructed from the pooled data, it is evident that the set of primers chosen for the study were able to detect the influence of growth regulators on the callus generated. Apparently the samples could be divided into two groups. Five samples viz., S5, S6, S7, S8 and S9 (mother plant) were genetically close to each other and hence formed a group. Another distinct group is formed by S2, S3 and S4. However S1 was found to be distinct from the two groups. Pairwise similarity computed for each method separately is given in Table 3. Among the callus from different samples,

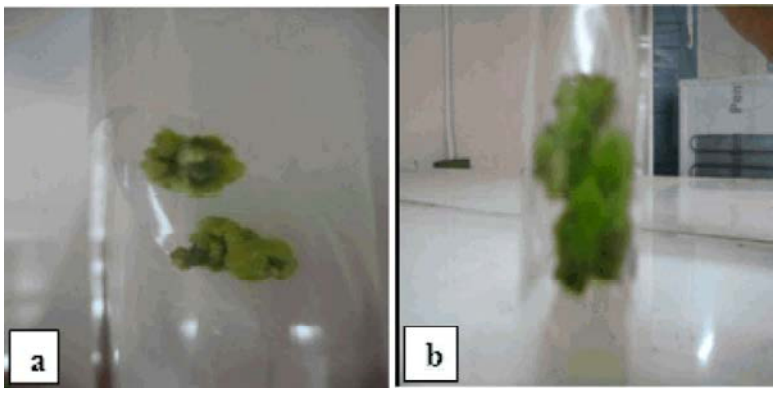


Fig. 1: Callus proliferation in MS media with hormonal concentration (a) IBA-0.4 mg/l and BAP-2.0 mg/l and (b) IBA-0.4 mg/l and BAP-2.5 mg/l

Table 2: Primers used for RAPD analysis and the band characteristics obtained for the callus from different hormonal combinations and mother plant

Primers	Sequences	No. of Total bands	No. of polymorphic bands	No. of monomorphic bands	Percentage of polymorphic bands (P%)	Total bands amplified
OPA05	AGGGGTCTTG	2	2	0	100.0	13
OPA11	CAATCGCCGT	3	2	1	66.6	23
OPC08	TGGACCGGTG	4	3	1	75.0	20
OPD07	TTGGCACGGG	2	2	0	100.0	16
OPD08	GTGTGCCCCA	4	4	0	100.0	13
OPE01	CCCAAGGTCC	7	6	1	85.7	39
OPE02	GGTGCGGGAA	3	3	0	100.0	12
OPH18	GAATCGGCCA	4	3	1	75.0	22
OPK12	TGGCCCTCAC	7	7	0	100.0	18
OPL05	ACGCAGGCAC	3	3	0	100.0	13
OPM12	GGGACGTTGG	2	2	0	100.0	9

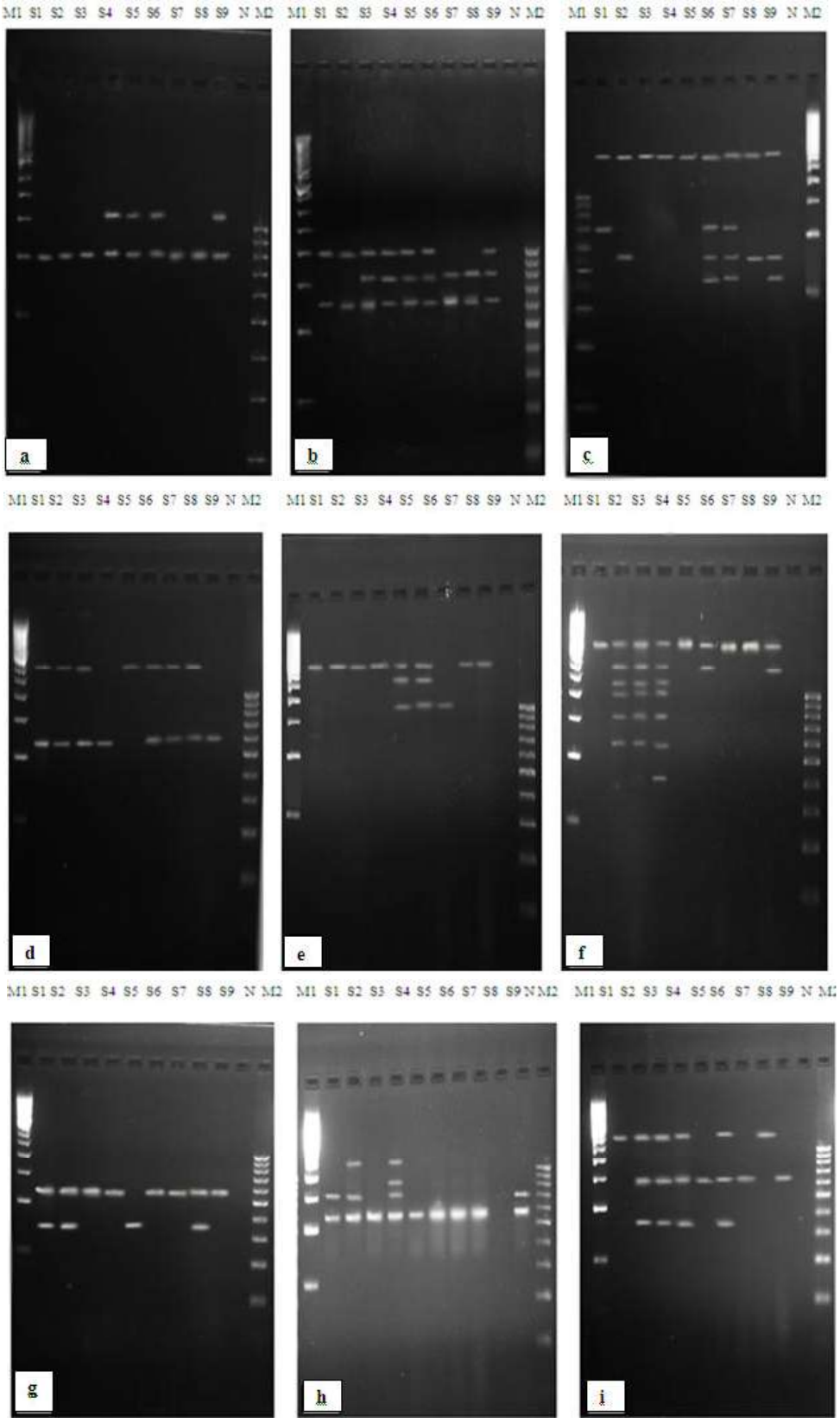


Fig. 2: Continued

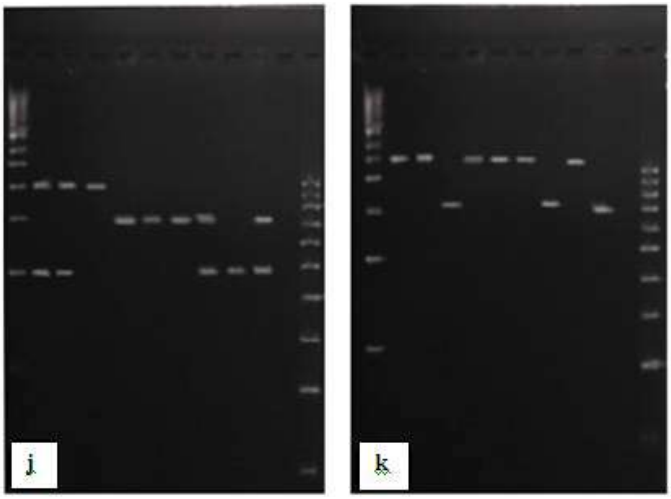
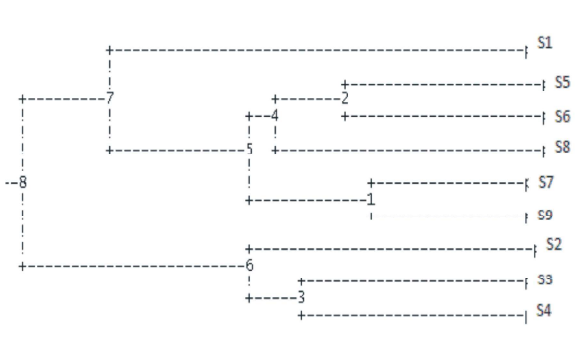


Fig. 2: RAPD pattern generated by primer OPA-05 (a), OPA-11 (b), OPC-08(c), OPD-07 (d), OPD-08 (e), OPE-01 (f). OPE-02 (g), OPH-18 (h), OPK-12 (i), OPL-05 (j) and OPM-12 (k). Lane M1 -DNA marker from Chromous Biotech (250 bp); Lanes S1 - S8: Amplicons of genomic DNA obtained from calluses from MS media with different hormonal combination; Lane S9: Amplicons of genomic DNA obtained from mother plant of *J. curcas*; Lane N: Negative control; Lane M2 -DNA marker from Step Up Genei (100bp) (See Table 1 for media composition)



Between	And	Length
8	7	5.17598
7	pop1	25.75557
7	5	8.67462
5	4	0.61467
4	2	4.07447
2	pop5	12.39181
2	pop6	12.39181
4	pop8	16.46627
5	1	7.72037
1	pop7	9.36058
1	pop9	9.36058
8	6	13.58799
6	pop2	17.34356
6	3	3.36431
3	pop3	13.97924
3	pop4	13.97924

Fig. 3: Dendrogram Based Nei's (1972) Genetic distance between the DNA samples of callus from MS media with different hormonal concentration and mother plant using UPGMA- Modified from NEIGHBOR procedure of PHYLIP Version 3.5. S1-S8 are the DNA samples of calluses from MS media with different hormonal concentration, S9 Genomic DNA obtained from mother plant of *J. curcas*.

Table 3: Nei's genetic identity (above diagonal) and genetic distance (below diagonal) between the DNA samples of callus from MS media with different hormonal concentration and mother plant. S1-S8 are the DNA samples of calluses from MS media with different hormonal concentration, S9 Genomic DNA obtained from mother plant of *J. curcas*

DNA samples	S1	S2	S3	S4	S5	S6	S7	S8	S9
S1	****	0.5854	0.4634	0.4634	0.5854	0.5122	0.5854	0.6585	0.6585
S2	0.5355	****	0.6829	0.7317	0.4634	0.4878	0.3659	0.6341	0.5366
S3	0.7691	0.3814	****	0.7561	0.5854	0.5610	0.4878	0.5610	0.6585
S4	0.7691	0.3124	0.2796	****	0.6341	0.6585	0.4878	0.6098	0.5610
S5	0.5355	0.7691	0.5355	0.4555	****	0.7805	0.6585	0.7317	0.6829
S6	0.6690	0.7178	0.5781	0.4177	0.2478	****	0.7317	0.7073	0.7561
S7	0.5355	1.0055	0.7178	0.7178	0.4177	0.3124	****	0.7317	0.8293
S8	0.4177	0.4555	0.5781	0.4947	0.3124	0.3463	0.3124	****	0.7073
S9	0.4177	0.6225	0.4177	0.5781	0.3814	0.2796	0.1872	0.3463	****

the highest similarity (0.78) was between callus from hormonal combination IBA- 0.4 mg/l and BAP- 2.5 mg/l (S6) and IBA- 0.4 mg/l and BAP- 2.0 mg/l (S5), while the least similarity (0.36) was between the callus from MS supplemented with IBA- 0.4 mg/l and BAP - 4.0 mg/l (S7) and IBA- 0.2 mg/l and BAP- 2.0 mg/l (S2) (Table 3). Similarly, the highest similarity with mother plant (0.82) showed by the callus from hormonal combination IBA- 0.4 mg/l and BAP- 4.0 mg/l (S7) where S2 (callus from IBA- 0.2 mg/l and BAP- 2.0 mg/l) showed the least similarity (0.53) with mother plant.

DISCUSSION

Fast growing, compact callus can be induced from MS medium, supplemented with various combinations of BAP and IBA. Vegetative plant parts especially leaves are desirable explants for *in vitro* cultures because plant regeneration from these explants would preserve the genetic identity of the parent genotype [16]. Callus induction is a prerequisite for large scale secondary metabolite production, adventitious improvement and for genetic engineering. The morphology and percentage of response varies according to the exogenous growth regulator given in the media. The texture of callus varies according to the nature of cytokinin and the auxin: cytokinin ratio [17].

Somaclonal variation is known to arise and cause genetic variability during the *in vitro* culturing of plant cells and tissues. Phytohormone treatments were effective in inducing genetic variability in *Saccharum officinarum* [18]. Plants regenerated from leaf base callus have shown variation at the DNA level during *in vitro* culture of *Curcuma longa* [19, 20]. Somaclonal variation reduces the commercial value of plants [21]. Thus it is important to detect somaclonal variation at an early stage of plant growth to avoid economic loss [2]. Cytological assessment has been proposed but not often used as it can be difficult in many species where chromosomes are difficult to observe. Analyses of secondary metabolites and isozyme patterns have also been used, but they are limited in their sensitivity [23]. It is therefore necessary to apply new approaches for studying the genetic variation in plants with related to phenotypic changes. One acceptable approach is to examine the genome of regenerants with the use of molecular markers [24].

In this study, total percentage of the polymorphism obtained was 90.24%. The common amplicons in the agarose profile differ only in the intensity of their bands in the RAPD spectra [25]. One of the possible explanations for this polymorphism could be a different copy number of the corresponding DNA loci in the samples under study [26].

The variation induced during *in vitro* culture condition may affect the regenerants of the same. In the present study, callus from the media MS with hormonal combination IBA- 0.4 mg/l and BAP- 4.0 mg/l (S7) showed the highest similarity with mother plant (0.82). Callus from MS media with hormonal combination IBA- 0.4 mg/l and BAP- 2.0 mg/l (S5), IBA- 0.4 mg/l and BAP- 2.5 mg/l (S6) and IBA- 0.6 mg/l and BAP- 2.0 mg/l (S8) were also similar to the mother plant (S9). These hormonal combinations are better suited for the micropropagation, secondary metabolite production and genetic engineering.

The sample S2 (callus from IBA- 0.2 mg/l and BAP- 2.0 mg/l) was found to show the least similarity (0.53) with mother plant. Callus formed in media MS with BAP alone (S1) grouped separately in the dendrogram. This indicates
some genetic changes that occur during the introduction into *in vitro* culture and continuous culturing due to genome rearrangement during early culturing, when cell is under stress to survive in the new environment. The treatment used in tissue culture, with high growth rate, may increase the variant numbers [27]. The callus samples S2, S3 and S4, which were grown in MS with hormonal combinations IBA- 0.2mg/l and BAP- 2.0 mg/l, IBA- 0.3mg/l and BAP- 1.0 mg/l and IBA- 0.3mg/l and BAP- 2.5 mg/l respectively showed less similarity with mother plant. These changes may be due to the sequence variations in loci revealed by the primers used in the donor plant DNA or may be due to sequences outside these loci which results in new amplicons appearing in addition to the majority of fragments characteristic of donor plant DNA.

It was reported that lower level of auxin greatly reduced variability in regenerated *Hordeum* plants, compared with a high concentration [28]. Contradictory to this, our study showed that low concentration of auxin contributes variation. And in the absence of auxin, change in the morphology of the callus was evident. In the dendogram drawn callus induced from lower level of auxin (S2, S3 and S4) and auxinless media grouped separately from samples, S5, S6, S7, S8 and motherplant. Thus in our study changes in auxin (IBA) level seem to affect genetic fidelity in the callus formed. According to Saieed *et al*. [29] this variation may be a consequence of the stress inherent in cellular deprogramming induced by plant growth regulators such as the synthetic auxin analog 2,4-dichlorophenoxy-acetic acid (2,4-D). So for

cultures with genetic stability, the exogenous auxin cytokinin stimulus provided by the callus maintenance medium should be necessarily studied and the concentration have to be standardized for each species. Epigenetic changes resulting in altered hormone response have been reported for several species [30]. Supporting this even a slight change in the hormonal concentration showed dissimilarities in callus with RAPD pattern of the motherplant.

The availability of growth hormones in the culture media regulates the genetic pattern. RAPD markers can be used to detect somaclonal variation in order to identify hormonal combinations and conditions to minimize the incorporation of genetic changes and to regenerate true to type plants.

CONCLUSION

The present study shows the morphological and genetic influence of different combinations of auxin (IBA) and cytokinin (BAP) supplementation of MS media on the callus generated. RAPD was effective in detecting variations that may occur as a result of callus induction from leaf explants of the of *J. curcas*. Four of the eight auxin cytokinin combinations tried were found to show similarity with the motherplant. Since callus culture system offer many advantages as a model system for several biological investigations the present findings could be used in mass propagation, secondary metabolite production and genetic engineering studies.

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