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RESEARCH ARTICLE

Plant regeneration through the direct induction of shoot buds from petiole explants of *Jatropha curcas*: a biofuel plant

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Genotype; *Jatropha curcas*; petiole; regeneration; thidiazuron.

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Abstract

An efficient and reproducible method for the regeneration of *Jatropha curcas* plants has been developed. The method employed direct induction of shoot buds from petiole explants, without the formation of an intervening callus using a Murashige and Skoog (MS) medium supplemented with different concentrations of thidiazuron (TDZ). The best induction of shoot buds (58.35%) and the number of shoot buds per explant (10.10) were observed when *in vitro* petiole explants were placed horizontally on MS medium supplemented with 2.27 μM TDZ after 6 weeks. The induced shoot buds were transferred to MS medium containing 10 μM kinetin (Kn), 4.5 μM 6-benzyl aminopurine (BAP) and 5.5 μM α-naphthaleneacetic acid (NAA) for shoot proliferation. The proliferated shoots could be elongated on MS medium supplemented with different concentrations and combinations of BAP, indole-3-acetic acid (IAA), NAA and indole-3-butyric acid (IBA). MS medium supplemented with 2.25 μM BAP and 8.5 μM IAA was found to be the best combination for shoot elongation and 3.01–3.91 cm elongation was achieved after 6 weeks. However, significant differences in plant regeneration and shoot elongation were observed among the genotypes studied. The orientation (horizontal or vertical) and source (*in vitro* or *in vivo*) of explants also significantly influenced plant regeneration. The elongated shoots could be rooted on half-strength MS medium supplemented with 2% sucrose, different concentrations and combinations of IBA, IAA and NAA, and 0.25 mg L^{-1} activated charcoal. Half-strength MS medium supplemented with 2% sucrose, 15 μM IBA, 5.7 μM IAA, 5.5 μM NAA and 0.25 mg L^{-1} activated charcoal was found to be the best for promoting rooting. The rooted plants could be established in soil with more than 90% survival.

Introduction

Global energy supply is based mainly on fossil fuels, which have many disadvantages, besides, their fast depletion. It is widely agreed that more sustainable alternative energy sources need to be developed in the near future. One potentially promising option consists of biofuels, which are renewable in nature and do not contribute to climate change (Openshaw, 2000; Mandpe *et al*., 2005). Many oil producing crops and plants have been considered for the purpose, among these *Jatropha curcas*, a member of *Euphorbiaceae* family has evoked considerable interest all over the tropics as a potential biofuel plant (Martin & Mayeux, 1985; Jones & Miller, 1991; Fairless, 2007; Ghosh *et al*., 2007). Since, *J. curcas* does not compete with conventional crops for cultivation, the dilemma of food verses fuel does not arise (Ghosh *et al*., 2007). *J. curcas* is primarily propagated through seeds, and significant variations in seed yield and oil content have been observed in plants raised through seeds (Pant *et al*., 2006; Jha *et al*., 2007). Seed viability and the rate of germination are low (Heller, 1996), and seed screening for quality is a

laborious task, thus propagation through seed may not provide enough high quality material for sustainable agriculture. It has also been observed that a large amount of seed is required for the raising of planting material. Alternative vegetative propagation techniques would not only help in raising high quality planting material, but would also allow seed to be diverted for biodiesel preparation. Propagation can also be carried out without the loss of traits by stem cutting. However, the limitation in generation of large scale planting material is (a) availability of sufficient quantity of material and (b) propagation is seasonal. Thus, conventional propagation through seeds is not reliable and vegetative propagation by stem cuttings is inadequate to meet the demand (Heller, 1996; Openshaw, 2000). Therefore, improvement programmes of *J. curcas* by modern methods of agro-biotechnology are of interest worldwide. This has increased the importance of developing tissue culture methods to facilitate large scale production of true-to-type plants and for the improvement of the species using genetic engineering techniques.

In vitro regeneration techniques offer a powerful tool for germplasm conservation, mass-multiplication of true-to-type plants and genetic transformation. Attempts have been made to regenerate *J. curcas* using different explants (Sujatha & Mukta, 1996; Wei *et al*., 2004; Sujatha *et al*., 2005; Jha *et al*., 2007; Rajore & Batra, 2007). All the above studies were either through callus-mediated regeneration or direct shoot morphogenesis with interspersed callus. Despite the regeneration systems achieved using different explants of *J. curcas*, the presence of intermediary callus or callus-mediated regeneration is least desired for the production of true-to-type plants. It has also been reported that regeneration in *J. curcas* is highly genotype dependent (da Camara Machado *et al*., 1997). From the literature, it is evident that a genotype independent regeneration protocol for *J. curcas* has not been reported. The petiole is a somatic tissue and plants raised from the petioles have been shown to be more resistant to genetic variation (Pierik, 1991).

Therefore, the objective of the present study was to develop an *in vitro* regeneration method without the requirement of an intervening callus, for mass propagation of true-to-type plants and genetic transformation. As far as we are aware, this is the first report of direct shoot bud induction from petiole explants. This study also compares the regeneration efficiency of source and orientation of explants.

Materials and methods

Plant material and source of explant

The best performing *J. curcas* genotypes (CSMCRI-JC-1, CSMCRI-JC-2, CSMCRI-JC-3 and CSMCRI-JC-4) identified in field experiments at Central Salt & Marine Chemicals Research Institute (CSMCRI), Chorvadla, India (21°75′N, 72°14′E) were selected for the present study. *In vitro* aseptic shoot cultures of these selected genotypes were established by culturing nodal explants collected from 3- to 4-year-old plants. The excised nodal explants of 3–4 cm with axillary buds were surface sterilised with 0.1% mercuric chloride ($HgCl_2$) for 15 min and rinsed five times in sterile distilled water. The sterilised nodal explants were cultured on Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with 3% sucrose, 27 μM 6-benzyl aminopurine (BAP) and 5 μM indole-3-butyric acid (IBA) for the sprouting of axillary buds and formation of shoots. After 4 weeks of culture, petioles were collected from young leaves of these axillary shoots and used as *in vitro* explants. For *in vivo* explants, petioles collected from leaves, which were close to apical buds from 3 to 4-year-old plants of the above selected genotypes, were sterilised with 0.1% mercuric chloride ($HgCl_2$) for 7 min separately and used as *in vivo* explants.

Shoot bud induction

The petioles from both *in vitro* and *in vivo* grown plants were cultured on MS medium supplemented with 3% sucrose and various concentrations of thidiazuron (TDZ) to establish the optimum culture medium for shoot bud induction. Petioles were inoculated on to the medium in 200 × 38 mm culture tubes (Borosil, India) both in the horizontal and vertical positions. The percentage of induction of shoot buds and the number of shoot buds per explant were recorded after 6 weeks of culture.

Shoot proliferation and elongation from induced shoot buds

The induced shoot buds were transferred on to MS medium supplemented with 3% sucrose, 10 μM kinetin (Kn), 4.5 μM BAP and 5.5 μM α-naphthaleneacetic acid (NAA) for shoot proliferation (Reddy *et al*., 2008). Shoots were individually separated and the number of shoots per petiole explant was recorded after 4 weeks. Individual shoots were further tested for their elongation on MS medium supplemented with 3% sucrose and different concentrations and combinations of BAP (2.25–4.5 μM), indole-3-acetic acid (IAA) (2.8–8.5 μM), NAA (2.75–8.25 μM) and IBA (2.5–7.5 μM). The length of the elongated shoots was recorded after 6 weeks of culture.

Rooting and acclimatisation

Green and healthy elongated shoots with three to four leaves were excised and cultured on half-strength MS

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medium supplemented with 2% sucrose and different concentrations and combinations of auxins, viz. IBA (5–15 μM), IAA (5.7–11.4 μM) and NAA (5.5–11 μM) for rooting. The percentage of root induction was recorded after 4 weeks. Rooted shoots were carefully taken out of the medium and washed thoroughly in sterile distilled water to remove MS medium attached to the roots. The plants were transferred to polythene bags containing sterilised sand and soil (1:1) and wetted with 0.02% w/v carbendazim and covered with transparent plastic bags to maintain humidity. After 3–4 weeks, the established plants were transferred to a greenhouse (temperature $25 \pm 3^{\circ}C$ and relative humidity 70–80%) for further growth and the number of surviving plants were recorded after an additional of 6–8 weeks.

Culture conditions and data analysis

Uniform culture conditions were applied in all experiments. The pH of the medium was adjusted to 5.7 using 1 N KOH or HCl, prior to autoclaving at 1.05 kg cm^{-2} pressure at 121°C for 20 min. The cultures were maintained at $25 \pm 2^{\circ}C$ under a 16-h photoperiod with light intensity of 35–40 μmol m^{-2} s^{-1} (cool white fluorescent tubes).

All the experiments were set up in a factorial completely randomised design (FCRD) and repeated three times with 25 replicates per treatment, with one explant per test tube. Data were subjected to analysis of variance (ANOVA), analysed by four factor FCRD analysis for shoot bud induction and two factor CRD analysis for shoot elongation and rooting using a BASIC software package (Anand Agricultural University, Gujarat, India) at the 5% probability level. The results were expressed as mean ± SE of three independent experiments.

Results

Effect of thidiazuron on shoot bud induction

The concentration of TDZ in the medium significantly influenced the response of shoot bud induction irrespective of genotype studied. The percentage of induction of shoot buds and the number of induced shoot buds per explant were directly proportional to the concentration of TDZ. Of the different concentrations of TDZ tested, the highest percentage of shoot bud induction (73.74%) and highest number of induced shoot buds (14.09) per explant were observed in the presence of 9.08 μM TDZ, among the genotypes studied. However, further proliferation and elongation of shoot buds were inhibited because of compact shoot bud induction at this concentration. It was observed that 2.27 μM TDZ was the optimum concentration for the induction of shoot buds and subsequent subculture. At 2.27 μM TDZ, the percentage of shoot bud induction varied from 33.19% to 58.35% and the number of induced shoot buds per explants varied from 4.18 to 10.10 among the genotypes (Tables 1 and 2).

Effect of orientation of explants on shoot bud induction

The orientation of explants significantly influenced the response of shoot bud induction at the tested concentrations of TDZ among the genotypes studied. The percentage of induction of shoot buds and the number of induced shoot buds per explant were higher in the horizontal position, as compared with the vertical position, irrespective of genotype. The percentage of induction of shoot buds varied from 4.87% to 73.74% in the horizontal position (Table 1; Fig. 1A and Fig. 1B) and 3.04–61.22% in the vertical position (Table 1; Fig. 1C and Fig. 1D). Whereas the number of induced shoot buds per explant varied from 2.84 to 14.09 in the horizontal position and 2.10–13.08 in the vertical position at the concentrations of TDZ tested among the genotypes (Table 2).

Effect of explant source on shoot bud induction

The source of explants also significantly influenced plant regeneration at the concentrations of TDZ tested among the genotypes studied. *In vitro* explants responded more efficiently than *in vivo* explants, irrespective of genotype. The percentage of induction of shoot buds varied from 4.86% to 73.74% for *in vitro* explants (Table 1; Fig. 1A and Fig. 1C) and 3.04–61.19% for *in vivo* explants (Table 1; Fig. 1B and Fig. 1D). Whereas the number of shoot buds induced per explant varied from 2.10 to 14.09 for *in vitro* explants and 2.17–13.00 for *in vivo* explants at the concentrations of TDZ tested among the genotypes (Table 2).

Effect of genotype on shoot bud induction

Significant differences in the percentage of induction of shoot buds and the number of induced shoot buds per explant were observed among the genotypes studied at the concentrations of TDZ tested. CSMCRI-JC-2 performed best at the concentrations of TDZ tested both in terms of the percentage of induction of shoot buds and the number of shoot buds per explant. The percentage induction of shoot buds in CSMCRI-JC-2 and CSMCRI-JC-1 varied from 8.01% to 73.74% and 3.04–62.13%, respectively (Table 1), and the number of induced shoot buds per explant varied from 4.03 to 14.09 and 2.20–11.21, respectively at the concentrations of TDZ tested (Table 2). The response of CSMCRI-JC-3 and CSMCRI-JC-4 was poor, for both *in vitro* and *in vivo* explants. The percentage of induction of shoot

Table 1 Effect of different concentrations of thidiazuron (TDZ), orientation (O) (horizontal or vertical), source (S) (*in vitro* or *in vivo*) and genotype (G) on the percentage of induction of shoot bud from petiole explants of four genotypes of *J. curcas*[a]

		In Vitro				*In Vivo*			
O	TDZ (μM)	CSMCRI-JC-1	CSMCRI-JC-2	CSMCRI-JC-3	CSMCRI-JC-4	CSMCRI-JC-1	CSMCRI-JC-2	CSMCRI-JC-3	CSMCRI-JC-4
Horizontal	0.22	6.71 ± 0.67	10.67 ± 1.11	5.89 ± 0.52	13.95 ± 1.12	4.87 ± 0.58	8.92 ± 0.98	5.01 ± 0.51	12.58 ± 1.07
	0.45	14.48 ± 1.01	21.21 ± 1.33	13.64 ± 0.67	14.26 ± 1.41	12.52 ± 0.86	19.67 ± 1.21	11.84 ± 0.71	14.08 ± 1.21
	0.90	21.73 ± 1.19	29.26 ± 1.72	19.03 ± 1.51	14.62 ± 1.52	19.13 ± 1.16	24.29 ± 1.43	18.12 ± 1.20	15.04 ± 1.75
	2.27	50.07 ± 1.98	58.35 ± 2.14	41.92 ± 1.17	43.06 ± 1.43	35.13 ± 1.16	46.19 ± 1.76	40.07 ± 1.46	38.65 ± 2.02
	4.54	59.88 ± 2.51	65.32 ± 2.57	49.53 ± 2.26	57.27 ± 2.32	44.08 ± 1.79	59.08 ± 1.78	48.17 ± 1.51	47.35 ± 1.91
	9.08	62.13 ± 2.83	73.74 ± 3.25	52.25 ± 1.85	59.65 ± 2.47	51.22 ± 2.41	61.19 ± 2.59	48.25 ± 2.30	48.91 ± 2.43
Vertical	0.22	5.70 ± 0.28	9.17 ± 0.60	4.86 ± 0.47	11.90 ± 0.45	3.04 ± 0.30	8.01 ± 1.09	4.06 ± 0.62	11.98 ± 0.93
	0.45	13.81 ± 0.70	18.46 ± 1.15	11.69 ± 0.88	13.38 ± 0.63	11.53 ± 0.42	17.61 ± 1.33	13.15 ± 0.77	13.31 ± 0.68
	0.90	19.47 ± 1.22	29.38 ± 1.43	19.80 ± 1.31	14.60 ± 1.32	17.73 ± 1.21	23.80 ± 1.18	19.15 ± 1.25	14.34 ± 1.55
	2.27	39.76 ± 1.89	51.19 ± 1.46	38.66 ± 2.58	39.66 ± 1.58	33.19 ± 1.53	43.55 ± 2.00	39.11 ± 1.32	37.54 ± 1.75
	4.54	48.60 ± 2.32	58.85 ± 2.85	46.54 ± 2.22	50.81 ± 2.27	41.34 ± 1.75	56.52 ± 2.37	41.08 ± 1.76	47.04 ± 2.34
	9.08	52.56 ± 2.34	61.22 ± 3.72	49.33 ± 2.56	55.58 ± 3.36	47.76 ± 1.83	58.32 ± 2.39	46.14 ± 2.33	51.38 ± 2.36

ANOVA Summary Table

Source	d.f.	MS	F
S	1	1113.75	123.52*
O	1	506.80	56.20*
S × O	1	112.14	12.43*
G	3	1298.82	144.05*
S × G	3	72.85	8.08*
O × G	3	27.99	3.10*
S × O × G	3	9.46	1.05 NS
TDZ	5	19,492	2161.82*
S × TDZ	5	92.72	10.28*
O × TDZ	5	47.07	5.22*
S × O × TDZ	5	17.50	1.94 NS
G × TDZ	15	93.97	10.42*
S × G × TDZ	15	14.88	1.65 NS
O × G × TDZ	15	5.72	0.635 NS
S × O × G × TDZ	15	6.45	0.716 NS
Error	192	9.01	
Total	287		

NS, not significant; ANOVA, analysis of variance.

[a]Data were taken after 6 weeks. Values represent means ± SE of 25 petiole explants per treatment in three repeated experiments.

*Significant at 5% probability level (*F* test).

buds in CSMCRI-JC-3 and CSMCRI-JC-4 varied from 4.06% to 49.33%, and 11.90–59.66%, respectively and the number of shoot buds induced per explant varied from 3.10 to 6.70 and 2.10–6.88, respectively at the concentrations of TDZ tested (Tables 1 and 2).

Shoot proliferation and elongation from induced shoot buds

The transfer of induced shoot buds from 0.22 to 2.27 μM TDZ containing medium to 10 μM Kn, 4.5 μM BAP and 5.5 μM NAA supplemented medium resulted in 2.01 ± 0.19 to 7.07 ± 0.89 shoots, whereas, higher concentration of TDZ (4.54–9.08 μM) resulted in 1.51 ± 0.22 to 4.53 ± 0.87 shoots in all genotypes studied (Fig. 1E). Individual (0.3–0.5 cm) shoots were separated from the clump of proliferated shoots and transferred to elongation medium containing different concentrations and combinations of plant growth regulators (PGRs) such as BAP, IAA, NAA and IBA (Table 3). Significant differences in elongation were observed at different concentrations and combinations of PGRs and genotypes. A combination of BAP and IAA was found to be the best for all the genotypes. The best elongation (3.01–3.92 cm) was observed on a medium containing 2.25 μM BAP and 8.5 μM IAA (Fig. 1F). The amount of elongation ranged from 1.88 to 3.91 cm on media containing combinations of BAP and IAA. Elongation was inhibited on medium containing BAP and IBA. The amount of elongation ranged from 2.00 to 2.44 cm on media containing combinations of

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Table 2 Effect of different concentrations of thidiazuron (TDZ), orientation (O) (horizontal or vertical), source (S) (*in vitro* or *in vivo*) and genotype (G) on the number of induction of shoot buds per petiole explant of four genotypes of *J. curcas*[a]

		In Vitro				*In Vivo*			
O	TDZ (μM)	CSMCRI-JC-1	CSMCRI-JC-2	CSMCRI-JC-3	CSMCRI-JC-4	CSMCRI-JC-1	CSMCRI-JC-2	CSMCRI-JC-3	CSMCRI-JC-4
Horizontal	0.22	3.19 ± 0.21	4.12 ± 0.24	3.54 ± 0.23	3.16 ± 0.27	3.07 ± 0.12	4.08 ± 0.12	3.39 ± 0.13	2.84 ± 0.11
	0.45	4.23 ± 0.32	6.55 ± 0.40	3.55 ± 0.44	4.35 ± 0.35	4.62 ± 0.18	7.32 ± 0.81	3.56 ± 0.17	4.01 ± 0.31
	0.90	5.71 ± 0.45	9.11 ± 0.51	4.63 ± 0.29	4.64 ± 0.20	5.09 ± 0.28	8.02 ± 0.88	4.09 ± 0.26	4.02 ± 0.29
	2.27	7.36 ± 0.35	10.10 ± 0.58	5.66 ± 0.36	5.53 ± 0.50	7.09 ± 0.43	9.43 ± 1.16	5.10 ± 0.62	5.73 ± 0.57
	4.54	9.14 ± 0.37	11.16 ± 0.58	5.81 ± 0.31	5.77 ± 0.30	8.49 ± 0.98	12.27 ± 0.76	5.30 ± 0.65	5.45 ± 0.83
	9.08	11.21 ± 0.61	14.09 ± 0.60	6.70 ± 0.43	6.88 ± 0.45	9.87 ± 0.61	13.00 ± 1.11	6.39 ± 0.77	6.03 ± 1.09
Vertical	0.22	2.20 ± 0.12	5.94 ± 0.97	3.67 ± 0.17	2.10 ± 0.12	3.04 ± 0.11	4.03 ± 0.43	3.10 ± 0.17	2.17 ± 0.17
	0.45	3.59 ± 0.22	7.95 ± 1.87	3.06 ± 0.15	3.07 ± 0.20	4.13 ± 0.52	4.25 ± 0.55	3.03 ± 0.40	3.67 ± 0.53
	0.90	4.90 ± 0.66	8.21 ± 1.98	4.52 ± 0.73	4.15 ± 0.36	4.15 ± 0.57	5.27 ± 0.76	4.08 ± 0.82	4.07 ± 0.94
	2.27	6.39 ± 0.87	9.65 ± 2.79	5.02 ± 0.84	4.26 ± 0.26	6.10 ± 1.10	6.99 ± 0.98	4.18 ± 0.45	4.23 ± 0.46
	4.54	9.43 ± 1.15	10.39 ± 2.59	5.40 ± 0.64	5.47 ± 0.29	7.06 ± 1.16	9.33 ± 1.47	5.04 ± 1.65	5.16 ± 0.66
	9.08	10.85 ± 0.71	13.08 ± 0.78	5.65 ± 0.60	5.84 ± 0.51	8.03 ± 2.03	11.30 ± 0.38	5.29 ± 1.17	5.13 ± 0.55

ANOVA Summary Table

Source	d.f.	MS	F
S	1	15.78	11.77*
O	1	27.12	20.27*
S × O	1	14.52	10.83*
G	3	253.52	189.12*
S × G	3	2.68	2.01 NS
O × G	3	0.59	0.44 NS
S × O × G	3	6.70	5.01*
TDZ	5	167.49	124.94*
S × TDZ	5	0.90	0.67 NS
O × TDZ	5	1.82	1.36 NS
S × O × TDZ	5	1.81	1.35 NS
G × TDZ	15	11.45	8.54*
S × G × TDZ	15	1.21	0.90 NS
O × G × TDZ	15	1.10	0.82 NS
S × O × G × TDZ	15	0.55	0.41 NS
Error	192	1.34	
Total	287		

NS, not significant; ANOVA, analysis of variance.

[a]Data were taken after 6 weeks. Values represent means ± SE of 25 petiole explants per treatment in three repeated experiments.

*Significant at 5% probability level (*F* test).

BAP and IBA, and 4.5 μM BAP and 7.5 μM IBA gave the best elongation (2.10–2.44 cm). Combinations of BAP and NAA promoted the lowest amount of elongation, with the elongation ranging from 1.01 to 1.99 cm. The best elongation was observed in CSMCRI-JC-2 (3.92 cm) followed by CSMCRI-JC-1 (3.52 cm) and CSMCRI-JC-4 (2.99 cm). The lowest amount of elongation was observed in CSMCRI-JC-3 (2.89 cm) (Table 3).

Rooting and acclimatisation

The percentage of rooting differed significantly depending upon the concentrations and combinations of auxins used, viz. IBA, IAA and NAA. Rooting increased with the increase in concentration of IBA and inclusion of IAA and NAA further increased the percentage of rooting (Table 4). The best rooting (51.05%) was observed on half-strength MS medium supplemented with 15 μM IBA, 5.7 μM IAA and 5.7 μM NAA after 4 weeks of incubation of harvested shoots (Fig. 1G). No significant differences were observed in the percentage of rooting among the genotypes. Following transfer to the rooted plants to polythene bags more than 90% of the plants survived. No visual morphological abnormalities were observed in regenerated plants (Fig. 1H and Fig. 1I).

Discussion

This is the first report on direct shoot bud induction from petiole explants of different genotypes of *J. curcas*

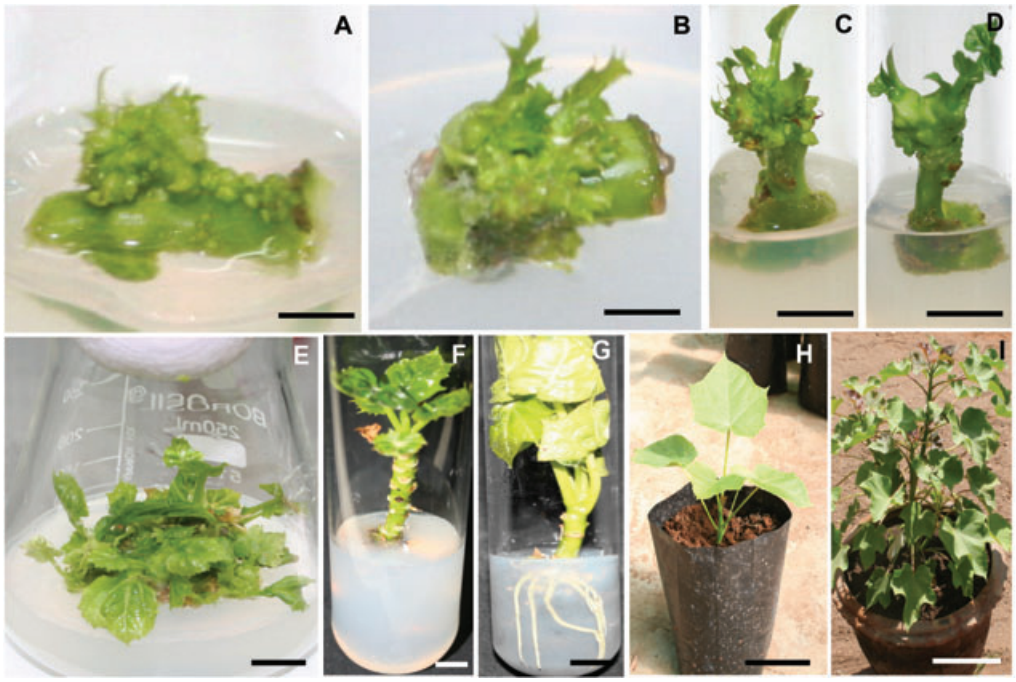

Figure 1 Direct induction of shoot buds from petiole explants of *J. curcas*. Direct induction of shoot buds from (A) *in vitro* petiole in horizontal position (bar 5 mm), (B) *in vivo* petiole in horizontal position (5 mm), (C) *in vitro* petiole in vertical position (bar 5 mm) and (D) *in vivo* petiole in vertical position on Murashige and Skoog (MS) medium with 2.27 μM thidiazuron after 6 weeks (bar 5 mm). (E) Shoot proliferation of induced shoot buds on MS medium with 10 μM kinetin + 4.5 μM 6-benzyl aminopurine (BAP) + 5.4 μM α-naphthaleneacetic acid (NAA) after 4 weeks (bar 100 mm). (F) Elongation of proliferated shoot on MS medium with 2.25 μM BAP and 8.5 μM indole-3-acetic acid (IAA) after 6 weeks (bar 1 mm). (G) Development of roots at the base of elongated shoot on half-strength of MS medium with 2% sucrose + 15 μM indole-3-butyric acid + 5.7 μM IAA + 5.5 μM NAA + 0.25 mg L-1 activated charcoal after 4 weeks (bar 1 mm). (H) Regenerated plants in polythene bags after 4 weeks (bar 100 mm). (I) A 6-month-old regenerated plant in pot under natural condition (bar 100 mm).

grown on MS medium containing TDZ. The concentration of TDZ in the medium, orientation, source of explants and genotype significantly influenced the regeneration response. Huetteman & Preece (1993) reported that TDZ is a potent cytokinin for woody plant tissue culture. In this study, the percentage response of explants forming shoot buds increased with an increase in the concentration of TDZ. Similar observations were reported in *Alstroemeria* species (Lin *et al*., 1997), *Solanum melongena* (Magioli *et al*., 1998), *Hagenia abyssinica* (Feyissa *et al*., 2005), *Embellia ribes* (Raghu *et al*., 2006) and *J. curcas* (Deore & Johnson, 2008). In the present investigation, it was observed that low concentrations of TDZ induced relatively fewer shoot buds, but these developed rapidly into shoots in subsequent culture. In contrast, media containing high concentration of TDZ had more visible primordia but, only a few were able to develop into shoots. Nielsen *et al*. (1993) also observed similar results in *Miscanthus sinensis*. In *Capsicum annuum*, 10 μM TDZ also induced multiple shoots which failed to elongate (Hyde & Phillips, 1996). The regeneration efficiency and number of shoot buds were higher in horizontally placed explants as compared with vertically placed explants irrespective of source of explants. Similar finding has been reported in *Heracleum candicans* (Sharma & Wakhlu, 2001) and *Eryngium foetidum* (Arockiasamy *et al*., 2002). This may be, because of the lack of surface contact of the explants to the medium in the vertical, as compared with the horizontal, position. Regeneration efficiency was also affected by explant source. *In vitro* explants had a higher rate of regeneration and number of shoot buds as compared with *in vivo* explants in both the horizontal and vertical positions, which may be because of differences in the concentration of endogenous growth regulators and their metabolism (Gray, 2004). Similar results were observed in *Echinacea purpurea* (Guda *et al*., 2003) and *Tomentosa steud* (Ozaslan *et al*., 2005). Genotype is one of the most important factors affecting regeneration (Feyissa *et al*., 2005). In our study, the genotypes showed differences in the percentage of induction of shoot buds, number of shoot buds per explant and elongation of regenerated shoot buds. Similar results were reported in *Morus alba* (Chitra & Padmaja, 2005) and *H. abyssinica* (Feyissa *et al*., 2005). Genotypic effects on shoot regeneration and elongation

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Table 3 Effect of plant growth regulators (PGRs) on elongation of proliferated shoots of four genotypes of *J. curcas*[a]

PGRs (μM)				Mean Shoot Length (cm)			
BAP	IAA	NAA	IBA	CSMCRI-JC-1	CSMCRI-JC-2	CSMCRI-JC-3	CSMCRI-JC-4
2.25	2.8	0	0	3.01 ± 0.19	3.11 ± 0.17	2.89 ± 0.38	2.99 ± 0.40
4.5	2.8	0	0	2.61 ± 0.17	2.73 ± 0.24	1.88 ± 0.15	2.12 ± 0.12
2.25	8.5	0	0	3.52 ± 0.15	3.91 ± 0.19	3.01 ± 0.52	3.11 ± 0.51
4.5	8.5	0	0	2.81 ± 0.19	2.94 ± 0.48	2.80 ± 0.34	2.74 ± 0.30
2.25	0	2.75	0	1.01 ± 0.11	1.11 ± 0.10	1.99 ± 0.11	1.67 ± 0.17
4.5	0	2.75	0	1.71 ± 0.12	1.82 ± 0.15	1.32 ± 0.15	1.11 ± 0.12
2.25	0	8.25	0	1.91 ± 0.17	1.88 ± 0.17	1.22 ± 0.12	1.11 ± 0.17
4.5	0	8.25	0	1.01 ± 0.10	1.10 ± 0.10	1.10 ± 0.11	0.99 ± 0.11
2.25	0	0	2.5	2.37 ± 0.24	2.17 ± 0.29	2.02 ± 0.12	2.16 ± 0.18
4.5	0	0	2.5	2.41 ± 0.29	2.11 ± 0.35	2.00 ± 0.13	2.15 ± 0.13
2.25	0	0	7.5	2.40 ± 0.23	2.17 ± 0.30	2.30 ± 0.17	2.17 ± 0.26
4.5	0	0	7.5	2.36 ± 0.37	2.12 ± 0.24	2.44 ± 0.24	2.10 ± 0.17

ANOVA Summary Table

Source	d.f.	MS	F
G	3	3.57	20.65*
PGRs	11	2.02	11.68*
G × PGRs	33	1.18	6.86*
Error	96	0.17	
Total	143		

BAP, 6-benzylaminopurine; NAA, α-naphthaleneacetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid.
[a]Data were taken after 6 weeks. Values represent means ± SE of 25 proliferated shoots per treatment in three repeated experiments.
*Significant at 5% probability level (*F* test).

Table 4 Effect of different concentrations and combinations of auxins with half-strength MS medium on the percentage of root induction in regenerated elongated shoots from petiole explants of four genotypes of *J. curcas*[a]

Auxins (μM)			Root Induction (%)			
IBA	IAA	NAA	CSMCRI-JC-1	CSMCRI-JC-2	CSMCRI-JC-3	CSMCRI-JC-4
5	0	0	8.21 ± 0.58	7.13 ± 0.71	6.11 ± 0.67	5.88 ± 1.17
10	0	0	12.13 ± 0.64	14.23 ± 0.58	10.09 ± 0.72	9.34 ± 1.37
15	0	0	17.10 ± 0.61	19.12 ± 1.16	14.13 ± 2.24	16.11 ± 0.72
5	5.7	5.5	20.21 ± 2.34	23.12 ± 1.66	22.11 ± 1.78	19.23 ± 1.19
10	5.7	5.5	22.18 ± 1.27	23.45 ± 1.33	22.05 ± 1.58	23.19 ± 1.18
15	5.7	5.5	51.05 ± 4.11	50. 23 ± 7.18	48.03 ± 4.55	49.25 ± 3.17
15	11.4	5.5	33.03 ± 2.25	32.01 ± 1.83	30.07 ± 3.86	33.76 ± 1.86
15	17.2	5.5	31.04 ± 1.79	33.12 ± 1.72	33.01 ± 2.28	34.04 ± 1.37
15	5.7	11	37.06 ± 1.68	35.92 ± 1.69	36.06 ± 1.73	38.01 ± 1.41
15	5.7	16.5	33.09 ± 1.80	32.11 ± 1.53	31.98 ± 1.65	30.23 ± 1.32

ANOVA Summary Table

Source	d.f.	MS	F
G	3	13.63	1.23 NS
Auxins	9	1987.42	180.03*
G × auxins	27	4.70	0.43 NS
Error	80	11.30	
Total	119		

NAA, α-naphthaleneacetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; NS, not significant.
[a]Data were taken after 4 weeks. Values represent means ± SE of 25 regenerated elongated shoots per treatment in three repeated experiments.
*Significant at 5% probability level (*F* test).

have been described in many species, and could be due, in part, to differences in the levels of endogenous growth regulators, particularly cytokinins during the induction period, although the precise mechanism remains unclear (Pellegrineschi, 1997; Schween & Schwenkel, 2003). Henry *et al*. (1994) reported that genotypic differences with respect to embryogenesis and regeneration result from quantitative or qualitative genetic differences. The inhibitory effects of high concentrations of TDZ on shoot elongation have been reported previously (Preece & Imel, 1991; Feyissa *et al*., 2005; Raghu *et al*., 2006), and our results are in agreement with above findings. Following the transfer of proliferated shoots to the elongation medium, the elongation of individual shoots depended upon the concentrations and combinations of PGRs in the medium. The maximum elongation was obtained using combinations of BAP and IAA as compared with BAP and IBA, and BAP and NAA. Our results are consistent with the previous reports (Christopher & Rajam, 1996; Venkataiah *et al*., 2003). Elongation was reduced in the medium containing BAP and IBA, which may be because of the proliferation of axillary buds. A similar observation has been reported in *Eupatorium triplinerve* (Martin, 2003). The low elongation observed in a medium containing BAP and NAA may be because of the profuse callusing at the basal end of proliferated shoots (Koroch *et al*., 2002; Kumar *et al*., 2008). The percentage of rooting was directly proportional to the concentration of IBA and a higher percentage was observed in combinations of auxins as compared with IBA alone. Similar observations have been observed in *Simmondsia chinensis* (Singh *et al*., 2008) and it is well established that auxins are potent hormones for rooting (Vuylasteker *et al*., 1998; Nandagopal & Kumari, 2007). The acclimatisation of the rooted shoots was easily accomplished and more than 90% of the plants were successfully transferred to polythene bags in under greenhouse conditions. No significant differences in rooting and subsequent hardening were observed for different genotypes studied.

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References

Arockiasamy S., Prakash S., Ignacimuthu S. (2002) Direct organogenesis from mature leaf and petiole explants of *Eryngium foetidum*. *Biologia Plantarum*, **45**, 129–132.

da Camara Machado A., Frick N.S., Kremen R., Katinger H., da Camara Machado M.L. (1997) Biotechnological approaches to the improvement of *Jatropha curcas*. In Proceedings of the International Symposium on Jatropha, p. 15. Managua, Nicaragua.

Chitra D.S., Padmaja G. (2005) Shoot regeneration via direct organogenesis from *in vitro* derived leaves of mulberry using thidiazuron and 6-benzylaminopurine. *Scientia Horticulturae*, **106**, 593–602.

Christopher T., Rajam M.V. (1996) Effect of genotype, explant and medium on *in vitro* regeneration of red pepper. *Plant Cell, Tissue and Organ Culture*, **46**, 245–250.

Deore A.C., Johnson T.S. (2008) High-frequency plant regeneration from leaf-disc cultures of *Jatropha curcas* L.: an important biodiesel plant. *Plant Biotechnology Reports*, **2**, 10–15.

Fairless D. (2007) Biofuel: the little shrub that could-may be. *Nature*, **449**, 652–655.

Feyissa T., Welander M., Negash L. (2005) *In vitro* regeneration of *Hagenia abyssinica* (Bruce) J.F. Gmel. (Rosaceae) from leaf explants. *Plant Cell Reports*, **24**, 392–400.

Ghosh A., Chaudhary D.R., Reddy M.P., Rao S.N., Chikara J., Pandya J.B., Patolia J.S., Gandhi M.R., Adimurthy S., Vaghela N., Mishra S., Rathod M.R., Prakash A.R., Shethia B.D., Upadhyay S.C., Balakrishna V., Prakash Ch.R., Ghosh P.K. (2007) Prospects for Jatropha methyl ester (biodiesel) in India. *International Journal of Environmental Studies*, **64**, 659–674.

Gray W.M. (2004) Hormonal regulation of plant growth and development. *PLoS Biology*, **2**, 1270–1273.

Guda C.D., Castello S., Savona M., Farina E. (2003) *Echinacea purpurea – in vivo* and *in vitro* propagation and field evaluation of ornamental clones. *Colture Protette*, **32**, 101–107.

Heller J. (1996) *Physic Nut,* Jatropha curcas *L. Promoting the Conservation and Use of Underutilized and Neglected Crops. No 1*. Rome, Italy: International Plant Genetic Resource Institute.

Henry Y., Vain P., Buyser J.D. (1994) Genetic analysis of *in vitro* plant tissue culture responses and regeneration capacities. *Euphytica*, **79**, 45–58.

Huetteman C.A., Preece J.E. (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell, Tissue and Organ Culture*, **33**, 105–119.

Hyde C.L., Phillips G.C. (1996) Silver nitrate promotes shoot development and plant regeneration of chile pepper (*Capsicum annuum* L.) via organogenesis. *In Vitro Cellular Developmental Biology-Plant*, **32**, 72–80.

Jha T., Mukherjee P., Datta M.M. (2007) Somatic embryogenesis in *Jatropha curcas* Linn., an important biofuel plant. *Plant Biotechnology Reports*, **1**, 135–140.

Jones N., Miller J.H. (1991) *Jatropha curcas*: a multipurpose species for problematic sites. *Land Resource Series*, **1**, 1–12.

Koroch A., Juliani H.R., Kapteyn J., Simon J.E. (2002) *In vitro* regeneration of *Echinacea purpurea* from leaf explants. *Plant Cell, Tissue and Organ Culture*, **69**, 79–83.

Kumar N., Pamidimarri S.D.V.N., Kaur M., Boricha G., Reddy M.P. (2008) Effects of NaCl on growth, ion accumulation, protein, proline contents, and antioxidant enzymes activity in callus cultures of *Jatropha curcas*. *Biologia*, **63**, 378–382.

Lin H.S., De Jeu M.J., Jacobsen E. (1997) Direct shoot regeneration from excised leaf explants of *in vitro* grown seedlings of *Alstroemeria* L. *Plant Cell Reports*, **16**, 770–774.

Magioli C., Rocha A.P.M., de Oliveira D.E., Mansur E. (1998) Efficient shoot organogenesis of eggplant (*Solanum melongena* L.) induced by thidiazuron. *Plant Cell Reports*, **17**, 661–663.

Mandpe S., Kadlaskar S., Degen W., Keppeler S. (2005) On road testing of advanced common rail diesel vehicles with biodiesel from the *Jatropha Curcas* plant. *Society of Automotive Engineering International*, **26**, 356–364.

Martin G., Mayeux A. (1985) Curcas oil (*Jatropha curcas* L.): a possible fuel. *Agriculture Tropical*, **9**, 73–75.

Martin K.P. (2003) Rapid axillary bud proliferation and ex vitro rooting of *Eupatorium triplinerve*. *Biologia Plantarum*, **47**, 589–591.

Murashige T., Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, **15**, 473–479.

Nandagopal S., Kumari R.B.D. (2007) Effectiveness of auxin induced *in vitro* root culture in chicory. *Journal of Central European Agriculture*, **8**, 73–79.

Nielsen J.M., Kirsten B., Hansen J. (1993) Long-term effects of thidiazuron are intermediate between benzyladenine, kinetin or isopentenyladenine in *Miscanthus sinensis*. *Plant Cell, Tissue and Organ Culture*, **35**, 173–179.

Openshaw K. (2000) A reviews of *Jatropha curcas*: an oil plant of unfulfilled promise, *Biomass Bioengineering*, **19**, 1–5.

Ozaslan M., Can C., Aytekin T. (2005) Effect of explant source on *in vitro* propagation of *Paulownia tomentosa* Steud. *Biotechnology and Biotechnological Equipment*, **19**, 20–26.

Pant K.S., Khosla V., Kumar D., Gairola S. (2006) Seed oil content variation in *Jatropha curcas* L. in different altitudinal ranges and site conditions in H.P. India. *Lyonia*, **11**, 31–34.

Pellegrineschi A. (1997) *In vitro* plant regeneration via organogenesis of cowpea [*Vigna unguiculata* (L.) Walp.]. *Plant Cell Reports*, **17**, 89–95.

Pierik R.L.M. (1991) Commercial aspects of micropropagation. In *Horticulture-New Technologies and Applications*, pp. 141–153. Eds J. Prakash and R.L.M. Pierik. Dordercht, the Netherlands: Kluwer Academic Publishers.

Preece J.E., Imel M.R. (1991) Plant regeneration from leaf explants of Rhododendron 'P.J.M. Hybrids'. *Scientia Horticulturae*, **48**, 159–170.

Raghu A.V., Geetha S.P., Martin G., Balachandran I., Ravindran P.N. (2006) Direct organogenesis from leaf explants of *Embelia ribes* Burm. – a vulnerable medicinal plant. *Journal of Forest Research*, **11**, 57–60.

Rajore S., Batra A. (2007) An alternative source for regenerable organogenic callus induction in *Jatropha curcas*. *Indian Journal of Biotechnology*, **6**, 545–548.

Reddy M.P., Kumar N., Vijayanand G., Singh A.H., Singh S. (2008) Method for micropropagation of *Jatropha curcas* plants from leaf explants (Patent filed US and PCT, Application No. 2537de2008).

Schween G., Schwenkel H.G. (2003) Effect of genotype on callus induction, shoot regeneration, and phenotypic stability of regenerated plants in greenhouse of *Primula* ssp. *Plant Cell, Tissue and Organ Culture*, **72**, 53–61.

Sharma R., Wakhlu A. (2001) Adventitious shoot regeneration from petiole explants of *Heracleum candicans* wall. *In Vitro Cellular Developmental Biology-Plant*, **37**, 794–797.

Singh A., Reddy M.P., Patolia J.S. (2008) An improved protocol for micropropagation of elite genotypes of *Simmondsia chinensis* (Link) Schneider. *Biologia Plantarum*, **52**, 538–540.

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

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

Venkataiah P., Christopher T., Subhash K. (2003) Thidiazuron-induced adventitious shoot bud formation and plant regeneration in *Capsicum annuum* L. *Journal of Plant Biotechnology*, **5**, 245–250.

Vuylasteker C., Dewaele S., Rambour S. (1998) Auxin induced lateral root formation in chicory. *Annals of Botany*, **81**, 449–454.

Wei Q., Lu W.D., Liao Y., Pan S.L., Xu Y., Tang L., Chen F. (2004) Plant regeneration from epicotyl explants of *Jatropha curcas*. *Journal of Plant Physiology and Molecular Biology*, **30**, 475–478.