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Influence of explant types and plant growth regulators on multiple shoot formation from *Jatropha curcas*

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ABSTRACT: *Jatropha curcas* is a potential non-edible biofuel-producing crop and is also important for its medicinal properties. The seed oil content varies from 4 to 40%, and thus the seed viability and rate of germination are low. Various explant types of *J. curcas* (stem, axillary bud, and shoot tip) were cultured on MS medium supplemented with different combinations 0.5 mg/l of BA and 0.05, 0.1, and 0.25 mg/l IBA. The results showed that MS medium supplemented with BA (0.5 mg/l) and IBA (0.25 mg/l) provided the highest number of shoots at 5.1, 5.3, and 5.25 per responding explant from stem, axillary bud and shoot tip explants, respectively, after 30 days of culture. Regenerated shoots were rooted on MS medium supplemented with 0.5 mg/l IBA after 30–40 days of culture.

KEYWORDS: embryos, hypocotyl, epicotyl

INTRODUCTION

Jatropha curcas L. (physic nut), a type of oil palm, is a potential non-edible biofuel-producing crop throughout the world[2]. *Jatropha* is a potential renewable energy crop as its oil can be used directly in a low speed diesel engine or upgraded via transesterification to conventional biodiesel[4]. At present, there is a need for elite planting material of *J. curcas* from government institutes, the private sector, and farmers for research, development and producing stem cuttings, seeds, oil and substitute energy. Techniques for culture in vitro need to be developed to generate material for germplasm preservation, rapid propagation, and crop improvement, which will increase field productivity and profitability leading to full supply of the demand for physic nuts, and future development[9]. In vitro techniques have been used for propagation of many plant species[8]. Tissue culture techniques offer rapid and continuous supply of the planting material. These techniques were undertaken to circumvent the problems associated with large scale multiplication of *J. curcas*[6]. Tissue culture propagated plants of *J. curcas* produce a better yield and yield-related traits[7] than seed-propagated plants. The aim of our study was to develop an appropriate method for the propagation of *J. curcas* in vitro. Here we report the effect of explants type and PGRs on multiple shoot formation in *J. curcas*.

MATERIALS AND METHODS

Plant materials

Seeds were cleaned with tap water and then soaked under running tap water for 8 h. The seeds were then gently cracked to expose the zygotic embryos surrounded by a kernel. The explants were immersed in 70% (v/v) ethanol for 5 min, in 20% (v/v) and 10% (v/v) Clorox (containing 0.02% Tween-20) for 10 and 20 min, respectively, followed by successive washing with sterile distilled water 3 times in a laminar flow station. The embryos were aseptically removed from the kernel and cultured on culture medium.

Induction of adventitious shoots

The embryos were germinated on growth-regulator-free Murashige and Skoog's (MS) medium[3]. The epicotyl explants taken from 15-day-old seedlings were separated and transferred to MS medium supplemented with 1–3 mg/l BA. After 30 days, shoot tips, axillary buds, and stems from epicotyl explants were cultured on the above medium supplemented with BA (0.5 mg/l), IBA (0.05, 0.1, and 0.25 mg/l), 30 g/l sucrose, and 7.5 g/l agar which we refer to as the multiplication medium. All combinations of PGR containing the medium were adjusted to pH 5.6

prior to adding agar and autoclaving at 121 °C, for 15 min. Shoots from the multiplication medium were separated individually and then transferred to MS medium containing 0.5 mg/l IBA as rooting medium. The cultures were maintained at 25 ± 2 °C under a 16-h photoperiod. Each treatment consisted of 20 explants with 2 replicates.

Induction of callus

Hypocotyl explants obtained from 15-day-old seedlings grown in vitro were used as initial explants for callus induction. The explants were cultured on MS medium supplemented with 3% sucrose and various concentrations of PGRs. The PGRs used in this experiment were BA, TDZ, 2iP, IBA, NAA, 2,4-D at concentrations of 1, 2, and 3 mg/l alone or in combination. The zygotic embryos were cultured on MS medium supplemented with BA (0.05, 1, 2, and 3 mg/l) in combination with IBA (0.1, 0.25, and 0.5 mg/l).

Statistical analysis

The experiments were set up in a completely randomized design. Data were analysed by analysis of variance to detect significant differences among the mean of treatments using Duncan's multiple range test (DMRT) at a 5% significance level.

RESULTS

Shoot induction from various explants

Epicotyl explants taken from 15-day-old seedlings started to grow and produce a single dominant shoot tip and 2–3 nodes along with the stem after culture for 30 days. All explants (shoot tips, nodes, and stem segments) cultured on MS medium supplemented with 0.5 mg/l BA and 0.25 mg/l IBA gave the best result in direct shoot bud formation. The number of shoots obtained from this medium was 5.21 per responding explant which was significantly different ($P < 0.05$) to other combinations. Among the explants tested, axillary buds gave the best results with the induction of 5.3 shoots per responding explant, followed by shoot tip (5.25) and stem explants (5.1). However, a significant difference was not obtained among those explants (Table 1, Figs. 1A–G). In the case of shoot bud induction through callus, fewer shoots were observed (Fig. 1H).

Callus and shoot induction from epicotyls and hypocotyls

The best multiple shoot formation and callus induction from both epicotyl and hypocotyl explants was obtained on culture medium supplemented with BA.

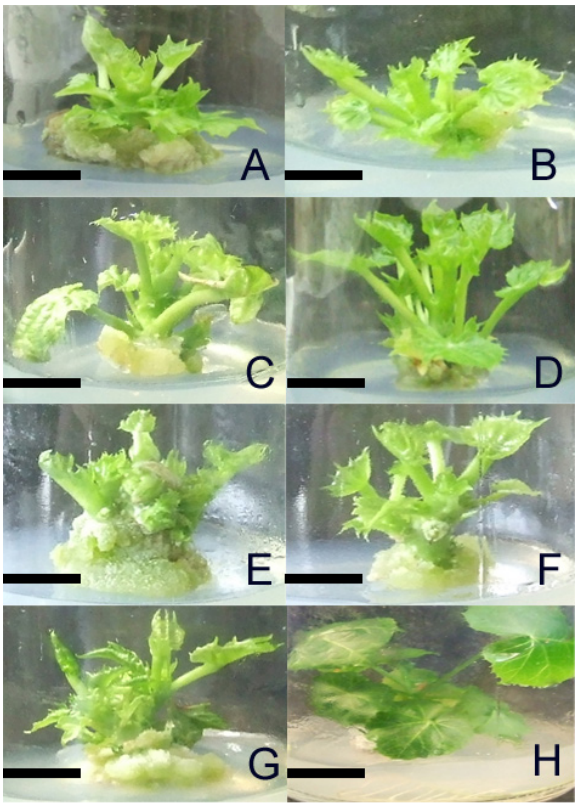

Fig. 1 Plant regeneration from various explants of *Jatropha curcas* L. A,B: axillary buds. C,D: shoot tips. E,F: stems. G: callus cultured on MS + 0.5 mg/l BA + 0.05–0.25 mg/l IBA for 30–50 days. H: Rooting from shoot cultured on MS + 0.5 mg/l IBA for 30 days (scale bar: 1 cm).

Callus and shoot regeneration formed when epicotyl explants were cultured on MS medium containing 1, 2, or 3 mg/l BA alone. Callus developed from the surface of explants (Fig. 2A) and characterized as white and green compact finally gave rise to multiple shoots on MS medium supplemented with 1, 2, and 3 mg/l BA. Epicotyl and hypocotyl explants cultured on MS medium containing 1, 2, and 3 mg/l TDZ, 2iP, IBA and NAA formed compact, white and green callus, without any shoot regeneration. IBA at a concentration of 1 mg/l was found to be the most suitable for callus induction (Fig. 2B). 2,4-D in all tested concentrations induced soft, friable, and light yellowish callus but without any shoot regeneration (Fig. 2C, Table 2).

Callus and shoot induction from embryos

Zygotic embryos produced different types of callus depending on the type of plant growth regulator used. Among the concentrations of BA tested (0, 0.05,

Table 1 Mean number of explants, which produced shoots after 30–50 days of culture on MS medium supplemented with various concentrations of plant growth regulators.

Conc. of plant growth regulator (mg/l)		No. of multiplied shoots/explants (shoots)			Mean
BA	IBA	stem	axillary bud	shoot tip	
0	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00
0.5	0.25	5.10 ± 1.33	5.30 ± 1.12	5.25 ± 0.91	5.21
0.5	0.1	4.10 ± 0.64^{a}	4.65 ± 1.08^{b}	4.90 ± 0.91^{c}	4.55
0.5	0.05	4.05 ± 0.68	4.10 ± 0.71	4.05 ± 0.88	4.06
0.5	0	3.70 ± 0.73^{a}	3.55 ± 0.75^{a}	3.50 ± 0.88^{a}	3.58
	Mean	4.23	4.40	4.42	
	F-test		*		
	C.V. (%)		20.72		

* Significantly different at $P < 0.05$ according to DMRT
Differing letters in the same row show that the values are significantly different by DMRT.

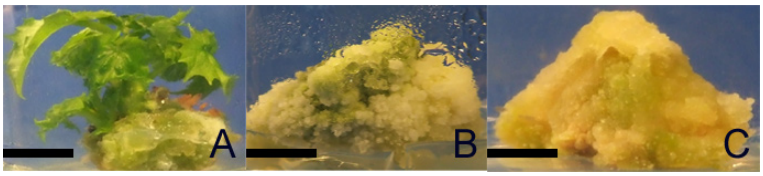

Fig. 2 Callus and shoot induction from epicotyls and hypocotyls. A: 1, 2, and 3 mg/l BA. B: TDZ, 2iP, IBA, and NAA. C: 1, 2, and 3 mg/l 2,4-D (scale bar: 1 cm).

0.25, 0.5, 1, 2, and 3 mg/l), all concentrations could induce both shoots and roots from all sides of the explant with little or no callus formation (Fig. 3A). The highest number of shoots induced was obtained on medium supplemented with 0.25 mg/l BA (Fig. 3B). In combination with IBA (0.1, 0.25, and 0.5 mg/l), BA (1, 2, and 3 mg/l) promoted callus formation (Fig. 3C). Increased concentrations of both BA and IBA gave a higher proliferation rate of callus in terms of fresh weight (Table 3). Morphological appearance of calluses obtained was similar. They were compact, white and green in colour. However, these calluses could not be regenerated into a complete plantlet.

Root formation

Individual shoots from multiplication medium were excised and successfully rooted on MS medium supplemented with 0.5 mg/l IBA. A high rate of root induction frequency of 60% was obtained after 30–40 days of culture.

DISCUSSION

This study demonstrated that MS medium supplemented with 0.5 mg/l BA and 0.25 mg/l IBA was the best PGR for induction of adventitious shoots directly from all explants. Similar observations were reported by Sujatha and Dhingra[5], Sujatha and Mukta[6] and Sujatha et al[7]. In general, the most effective explant for production of direct shoot bud formation are organized explants, e.g., shoot tips, axillary buds, and zygotic embryos. The present study has clearly shown that axillary buds gave the highest number of shoots (5.3 shoots/responding explant), whereas previous studies were unable to use this kind of explant for clonal

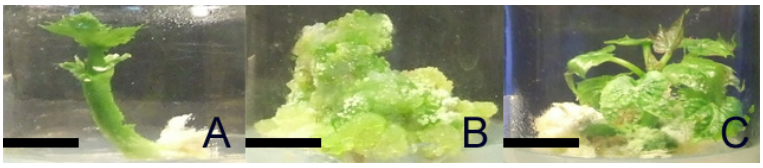

Fig. 3 Callus and shoot induction from embryos after 15 days. A: MS + 0, 0.05, 0.25, 0.5, 1, 2, and 3 mg/l BA. B: MS + 0.25 and 0.5 mg/l BA + 0.1, 0.25, and 0.5 mg/l IBA. C: MS + 2 and 3 mg/l BA + 0.1, 0.25, and 0.5 mg/l IBA (scale=1 cm).

Table 2 The influence of cytokinins and auxins on callus induction frequency from epicotyl and hypocotyl explants of *J. curcas*.

Conc. of plant growth regulator (mg/l)				Regeneration	
Cytokinin		Auxin		epicotyl	hypocotyl
	0		0	-	-
BA	1		0	S, G	S, G
	2		0	S, G	S, G
	3		0	S, G	S, G
TDZ	1		0	G	G
	2		0	G	G
	3		0	G	G
2iP	3		0	W	W
	-	IBA	1	G	G
	-		2	G	G
	-		3	G	G
	-	NAA	1	G	G
	-		2	G	G
	-		3	G	G
	-	2, 4-D	1	Y, F	Y, F
	-		2	Y, F	Y, F
	-		3	Y, F	Y, F

S = induced shoot, G = white and green compact callus, W = white compact callus, Y = light yellowish callus, F = soft friable callus.
Data recorded after 15 days of culture. Mean values were derived from twenty explants.
Mean with different letters were significantly different ($P < 0.05$) according to DMRT.

Table 3 The influence of BA and IBA on callus induction frequency and regeneration (R) from embryo cultures of *Jatropha curcas* L.

Conc. of plant growth regulator (mg/l)		Fresh weight (g)	Morphology of callus	R
BA	IBA			
0	0	0.00 ± 0.00	-	SR
0.05	-	0.00 ± 0.00	-	SR
0.25	-	0.00 ± 0.00	-	SR
0.25	0.1	1.85 ± 0.07^{a}	G	SR
0.5	-	0.00 ± 0.00	-	SR
0.5	0.1	1.87 ± 0.96^{a}	G	SR
0.5	0.25	$2.00 \pm .0.93^{a}$	G	SR
1	-	0.00 ± 0.00	-	SR
1	0.1	1.69 ± 0.08^{b}	G	-
1	0.25	2.86 ± 0.10^{bc}	G	-
1	0.5	3.06 ± 0.11^{cd}	G	-
2	-	0.00 ± 0.00	-	SR
2	0.1	3.05 ± 0.09^{cd}	G	-
2	0.25	3.04 ± 0.10^{cd}	G	-
2	0.5	3.32 ± 0.09^{de}	G	-
3	-	0.00 ± 0.00	-	SR
3	0.1	3.40 ± 0.10^{e}	G	-
3	0.25	3.45 ± 0.10^{e}	G	-
3	0.5	3.18 ± 0.08^{de}	G	-
F-test		*		
C.V. (%)		20.36		

G = white and green compact callus, SR = shoot and root.
Data recorded after 15 days of culture. Mean values were derived from twenty explants.
Values with different letters were significantly different ($P < 0.05$) according to DMRT.

propagation[7]. In addition, the number of organized explants is limited due to the limitation of those explants from mother plants; generally, one seedling produces only one shoot and cotyledonary node. So unorganised tissue, especially leaf, hypocotyl, stem, and petriole are alternative explants for direct shoot bud formation in *Jatropha* spp.

Several authors have reported direct shoot bud formation from cultured leaves on MS medium in the presence of the aforementioned PGR. However, we did not carry out any experiments using leaf as an explant source for direct shoot bud formation. In addition to leaf explant, hypocotyl, stem, and petiole have also been successfully exploited for shoot bud regeneration studies in vitro. Culture of stem segments in this present study provided direct shoot formation at 5.1 shoots per responding explant. Stem segments responded to 0.5 mg/l IBA whereas Sujatha and Mukta[6] reported an optimum result with a medium containing 0.1 mg/l IBA. The different response may be due to the different preparation of seedlings before culturing. In our case, we have used the seeds grown on PGR-free-MS medium whereas Sujatha and Mukta[6] used the seeds grown in vermiculite pots raised in a greenhouse (ex vitro conditions). The culture environment in which the explant was grown until explant sterilization might affect the balancing of the internal PGR of the explant[6].

Callus induction was obtained from cultured hypocotyls or zygotic embryos. The explant-produced callus could induce very few shoots and these were either scattered sparsely all along the explants or localized to a few areas and accompanied by callus formation. Plantlet regeneration from callus is generally influenced by a combination of auxin and cytokinin. Similar results have been reported[2,5]. Low concentrations of BA (0.5 mg/l) and IBA (0.5 mg/l) were necessary for differentiation of the shoot bud. High concentrations of BA and IBA (1.0 mg/l each)

were necessary for shoot elongation. In the case of hypocotyl explants that proliferate callus in the present study, shoot bud formation was obtained on medium supplemented with 1–3 mg/l BA alone, whereas zygotic embryo-derived callus regenerated shoot bud on low concentration of BA (0.05–0.5 mg/l) alone or in combination with IBA (0.1–0.25 mg/l). The differential response of these two explants may be attributed to the correlative interaction between the type of tissue and the concentration of endogenous growth regulators. In some cases, elongated shoots on proliferation medium produced spontaneous roots without being subjected to root induction medium[5]. In the present study, excised single shoots were rooted in rooting medium supplemented with 0.5 mg/l IBA for 30–40 days as in Ref. 1.

This study revealed that axillary buds from cotyledonary nodes were the best explants for micropropagation through direct induction of adventitious shoots when cultured on MS medium in combination with BA (0.5 mg/l) and IBA (0.25 mg/l). The regenerants obtained from this protocol seem to be normal and are under evaluation for their clonal uniformity.

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