BIOLOGIA PLANTARUM 54 (2): 369-372, 2010

BRIEF COMMUNICATION

Direct shoot regeneration from leaf explants of *Jatropha curcas* in response to thidiazuron and high copper contents in the medium

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Abstract

Leaf explants of *Jatropha curcas* cultured on Murashige and Skoog's (MS) medium supplemented with thidiazuron (TDZ; 0.90 μM) in combination with indole-3-butyric acid (IBA; 0.98μM) produced adventitious shoot buds directly on the surface of the explants without formation of intervening callus while shoot bud formation was accompanied with callus formation on medium supplemented with 6-benzylaminopurine (BAP; 13.3 μM) and IBA (2.46 μM). TDZ treatment resulted in more than twice higher rate of shoot bud induction than BAP. Shoot buds were multiplied and elongated following repeated transfers to medium containing BAP (2.22 μM) and gibberellic acid (GA_3; 1.44 μM). The effect of copper sulphate on differentiation of shoot buds from leaf segments was also investigated. Both shoot induction and multiplication media were supplemented with different levels of $CuSO_4$ (0 - 5 μM). Significant improvement in shoot bud induction was observed when the concentration of $CuSO_4$ was increased to 10 times the normal MS level. Healthy elongated shoots were rooted on half strength MS medium supplemented with IBA (2.46 μM). Rooted plantlets were transferred to field and survived. Histological analysis revealed direct formation of shoot buds from leaf explants.

Additional key words: adventitious bud regeneration, benzylaminopurine, gibberellic acid, indole-3-butyric acid, mineral nutrition.

The use of vegetable oil as biofuel is being considered as a suitable alternative to limited fossil fuel reserves. In this context, non-edible vegetable oil of *Jatropha curcas* has the potential of providing a promising and commercially viable alternative to petro-diesel (Gubitz *et al.* 1999). *Jatropha curcas* (family: *Euphorbiaceae*), is a multipurpose, drought resistant, perennial plant of Latin American origin, but it is now widespread throughout the tropical regions of the world (Openshaw 2000). Seeds are genetically heterozygous as *Jatropha* is cross pollinated, which results in a high degree of variation (Ginwal *et al.* 2005). To meet the large scale demand and ensure easy supply of the elite plant material, there is need to establish mass multiplication techniques. Though there are reports regarding the effect of different concentrations and combinations of plant growth regulators on regeneration from various explants in *J. curcas* (Sujatha and Mukta 1996, Datta *et al.* 2007, Deore and Johnson 2008), attempts have not been made to standardize the nutrient requirement to improve regeneration. Primarily, the composition and ratios of plant growth regulators are manipulated to optimize the quality and number of organs initiated (Ramage and Williams 2002). However, a tissue growth rate and the extent and quality of morphogenic response are also influenced by the type and concentration of nutrients supplied in the culture medium (Niedz and Evens 2007). In the present investigation, we have developed an efficient regeneration protocol by standardizing the copper requirements for improved shoot bud induction.

Young and fully-expanded leaves from apex were collected from 2-years-old mature *Jatropha curcas* L. tree growing in the University campus. The explants were washed under running water for 30 min, rinsed with 20 % *Extran* (*Merck*, Mumbai, India) followed by further washing under running water to remove traces of the detergent. These were then surface-sterilized with 4 % (m/v) NaOCl for 15 min followed by four consecutive

Received 18 August 2008, *accepted* 28 January 2009.

Abbreviations: BAP- 6-benzylaminopurine; GA_3 - gibberellic acid; IBA-indole-3-butyric acid; MS - Murashige and Skoog; PM - proliferation medium; SIM - shoot induction medium; TDZ - thidiazuron.

Acknowledgement: Varsha Khurana-Kaul thanks CSIR, New Delhi for the award of JRF/SRF.

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washings of sterile distilled water. Leaves were cut into 1.0 cm^2 segments and placed with abaxial side in contact with the medium. Murashige and Skoog (1962; MS) basal medium supplemented with 3 % sucrose, thidiazuron (TDZ) or 6-benzylaminopurine (BAP) in combination with indole-3-butyric acid (IBA), gelled with 0.8 - 0.9 % (m/v) agar (bacteriological grade, *Merck*), pH adjusted to 5.8 before autoclaving at 121 ºC and 1.2 - 1.3 kg cm^{-2} pressure for 20 min was used for all experiments. The cultures were incubated at 26 ± 1 ºC in a growth chamber with a irradiance of 25 µmol m^{-2} s^{-1} provided by white fluorescent tubes (*Philips*, Mumbai, India) and exposed to 16-h photoperiod. Thirty replicates were used for each treatment. Weekly observations were recorded.

During the first set of experiments, leaf segments were cultured on MS medium supplemented either with TDZ (0.90 - 22.72 µM) or BAP (2.22 - 22.22 µM) in combination with IBA (0.98 - 2.46 µM) for the induction of adventitious shoot buds. After 6 weeks of culture, shoot buds induced on the surface of leaf segments were multiplied by subculture on MS + TDZ (0.90 µM) + IBA (0.98µM) irrespective of the induction medium and elongated by repeated subculture at 2-week interval on medium containing BAP (2.22 µM) and gibberellic acid (GA_3, 1.44 µM). After the establishment of optimized basal media for induction of direct shoot buds from leaf segments (shoot induction medium, SIM), the next stage experiment was designed to study the effect of different concentrations of micronutrient $CuSO_4$ on induction as well as proliferation of shoot buds. Different concentrations of copper (0.1 to 5 µM) were added in MS medium along with TDZ and IBA. The control induction medium comprised of SIM along with 0.1 µM $CuSO_4$ (already present as micronutrient in MS basal medium). After 6 weeks of culture, the responding explants were sectored (clump of 3 - 4 shoot buds) and transferred to normal shoot proliferation medium (PM1, having MS level of $CuSO_4$) as well as modified shoot proliferation medium (PM2, having various concentration of $CuSO_4$). Weekly observations were recorded for 6 weeks.

Regenerated shoots, approximately 2 cm in length were separated individually and transferred to root induction medium consisting of half strength MS supplemented with 2.46 µM IBA. Plantlets with well-developed shoot and roots were taken out and washed gently to remove traces of agar and transferred to earthen pots containing garden soil and organic manure. High air humidity was maintained by covering the pots with polythene bags.

The leaf explants swelled and enlarged in 15 d of culture and started differentiating multiple shoot buds directly within 6 weeks of culture on the induction medium. Although the adventitious shoot buds were observed from entire leaf lamina, response was better on the mid-rib portion. Leaf explants responded to both cytokinins but TDZ in combination with IBA was more efficient in shoot bud regeneration than BAP (Table 1). BAP with IBA enunciated callusing along with shoot bud induction. Maximum induction of shoot buds was achieved on MS + 0.90 µM TDZ + 0.98 µM IBA (SIM) (Table 1). Repeated subculture of leaf segment to medium supplemented with 2.22 µM BAP and 1.44 µM GA_3 led to further differentiation and elongation of shoot buds. Histological analysis revealed direct formation of shoot buds from leaf explants (Fig. 1*C*). The abaxial epidermal cells increased in size and simultaneously divided into several planes which formed meristemoids on the surface of the midrib. These meristemoids, by further cell divisions gave rise to small protrusions of tissue, which gradually organized into shoot buds similarly as described by Saritha and Naidu (2008) in *Spilanthes acmella*.

The copper concentrations highly influenced the

Table 1. Effect of different combinations of plant growth regulators on direct shoot bud induction from cultured leaf segments of *Jatropha curcas*. Culture period: 6 weeks. Means ± SE, $n = 15$.

BAP [µM]	TDZ [µM]	IBA [µM]	Response [%]	Number of buds [explant^{-1}]
2.22	-	2.46	78	6.8 ± 0.4
4.44	-	2.46	73	4.2 ± 0.4
8.88	-	2.46	76	5.6 ± 0.2
13.33	-	2.46	80	8.6 ± 0.5
22.22	-	2.46	65	2.8 ± 0.4
-	0.90	0.98	88	18.8 ± 0.6
-	2.27	0.98	77	11.2 ± 0.6
-	4.54	0.98	70	7.2 ± 0.4
-	9.09	0.98	85	14.2 ± 0.7
-	13.63	0.98	64	8.8 ± 0.4
-	22.72	0.98	50	5.0 ± 0.3

Table 2. Effect of various concentrations of $CuSO_4$ on shoot bud induction from leaf segments of *Jatropha curcas* and on proliferation of shoot buds into shoots (0.5 - 1.0 cm) in second stage subculture. Culture period: 6 weeks. Means ± SE, $n = 15$.

$CuSO_4$ in SIM [µM]	Response [%]	Number of buds [explant^{-1}]	$CuSO_4$ in PM [µM]	Number of shoots [explant^{-1}] total	≥ 0.5 cm
0	65	10.2 ± 0.7	-	-	-
0.1	85	16.8 ± 0.6	0.1	5.4 ± 0.2	1.0 ± 0.3
0.5	74	15.6 ± 0.8	0.1	7.4 ± 0.7	1.0 ± 0.3
			0.5	10.4 ± 0.5	1.4 ± 0.2
1.0	80	22.8 ± 0.8	0.1	12.4 ± 0.5	2.0 ± 0.3
			1.0	20.8 ± 0.6	4.2 ± 0.4
2.0	67	14.8 ± 0.6	0.1	10.8 ± 0.6	2.0 ± 0.5
			2.0	12.0 ± 0.9	2.4 ± 0.2
3.0	60	19.2 ± 0.6	0.1	7.4 ± 0.7	1.6 ± 0.4
			3.0	8.8 ± 0.4	3.4 ± 0.5
5.0	55	6.4 ± 0.9	0.1	1.6 ± 0.2	0.6 ± 0.2
			5.0	1.8 ± 0.2	0

frequency and onset of shoot bud regeneration (Table 2).The number of adventitious shoot buds ranged from 6 to 23 on different Cu concentrations. There was a concomitant increase in number of shoot buds and reduction in time of induction with increasing amounts of Cu in the medium till optimum (1 µM; 10 times higher than in MS) (Fig. 1*B*,*D*). Shoot-bud formation frequency was 1.5 folds higher at 1 µM copper as compared to the control, but 5µM Cu inhibited shoot bud formation. Shoot buds formed in primary induction media were sectored and subcultured on PM1 and PM2 medium. Further multiplication was carried out and some well defined shoots (> 0.5 cm length) with emerging leaf primordia were observed (Table 2). Proliferation of shoots improved when they were transferred to PM2 medium containing higher Cu concentrations. Maximum shoot proliferation was obtained when shoot buds were induced and subcultured on medium with 1 µM Cu (Fig. 1*E*). Although PM2 medium induced proliferation of shoot buds, elongation was restricted on this medium, therefore, shoot buds were repeatedly subcultured on MS + 2.22 µM BAP + 1.44 µM GA_3 for elongation (Fig. 1*F*). Healthy elongated shoots were rooted successfully on half strength MS medium fortified with 2.46 µM IBA (Fig. 1*G*).

The type of culture vessel used had profound effect on the shoot bud differentiation from leaf segments. Nodular structures which failed to differentiate into well defined shoot buds were obtained when cultures were raised in 100 cm^3 Erlenmeyer flask (Fig. 1*A*), but in Petri dishes well differentiated shoot buds were obtained.

Leaf has been an explant of choice in micro-propagation of *J. curcas* in earlier reports also (Sujatha and Mukta 1996, Deore and Johnson 2008) as it is best suited for adventitious shoot regeneration and

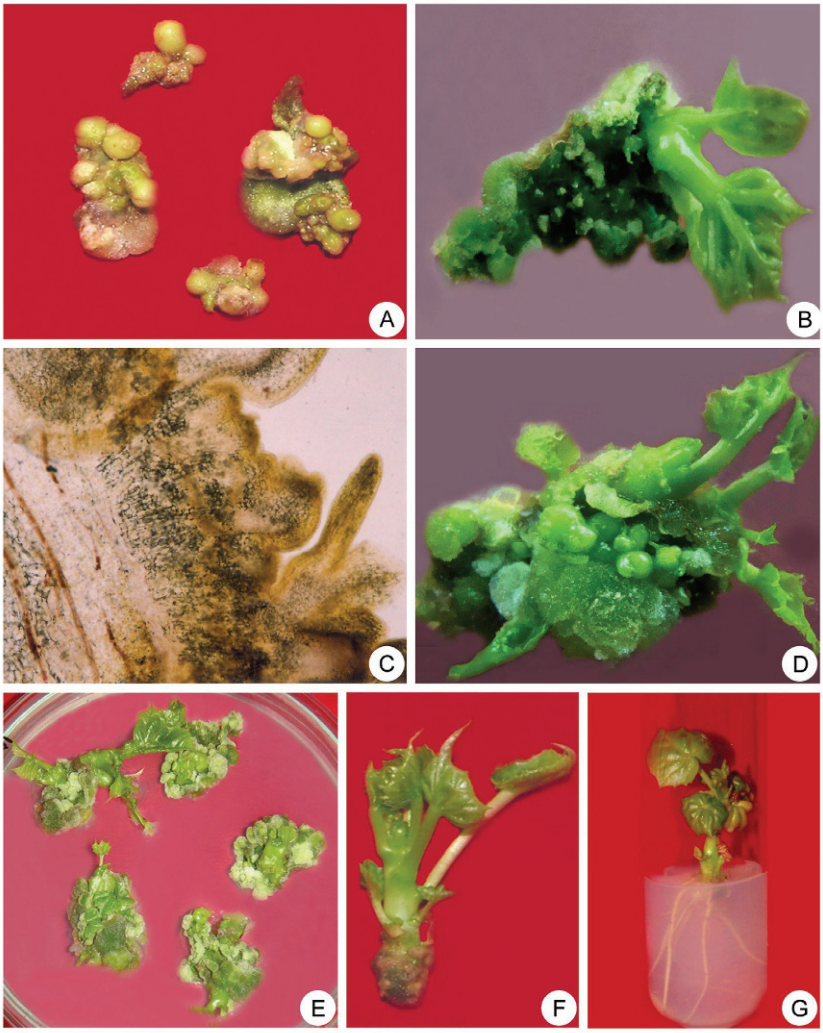

Fig. 1. Adventitious shoot bud induction from leaf segments of *Jatropha curcas*. *A* - Induction of nodular structures on MS + TDZ (0.90 µM) + IBA (0.98 µM) in flask; *B* - induction of shoot buds on the same medium but in Petri dish; C - histological analysis of the shoot buds differentiated along the mid rib portion of leaf; *D* - induction of shoot buds on the same medium but in the presence of 10 × higher concentration of $CuSO_4$ in the medium (1.0 µM); *E* - proliferation of shoot buds upon subculture to the medium with 1.0 µM $CuSO_4$; *F* - elongation of shoot buds on MS + BAP (2.22 µM) + GA_3 (1.44 µM); *G* - rooting of elongated shoot on half-strength MS + IBA (2.46 µM).

Agrobacterium mediated transformation experiments. Adventitious shoot formation is a reliable technique for clonal propagation as it prevents somaclonal variations. This point is of major importance because plants produced by direct organogenesis may exhibit greater genetic stability than those produced from callus (D'Amato 1975). In the present investigation we have used leaf explants for the induction of adventitious shoot buds. TDZ was found to be superior to BAP in elicitation of morphogenic response. It has been shown to exhibit stronger effects on shoot induction than conventional cytokinins over a wide range of species (D'Onofrio and Morini 2005, Radhika *et al.* 2006, Sujatha and Kumari 2007, Pavingerová 2009).

In *J. curcas*, optimization of Cu concentration in the medium significantly favoured the induction of shoot buds. Time required for induction of shoot buds was considerably reduced. This observation is supported by various reports in a range of monocotyledonous and dicotyledonous plants including *Triticale* (Purnhauser and Gyalai 1993), barley (Dahleen 1995), wheat (Tahiliani and Kothari 2004), *Capsicum* (Joshi and Kothari 2007), *Paspalum* (Kothari-Chajer *et al.* 2008) and *Stevia* (Jain *et al*. 2009). Copper is the component or activator of many enzymes involved in electron transport, protein and sugar (Purnhauser and Gyulai 1993) synthesis, is an essential cofactor of superoxide dismutase, cytochrome *c* oxidase, amino oxidase, laccase, plastocyanin and polyphenol oxidase (Clemens 2001).

In conclusion, present protocol advocates the use of enhanced concentration of copper in MS basal medium to improve regeneration in *Jatropha* cultures and to reduce the time for differentiation. Various other reports with manipulated levels of nutrient showed that every species has its own nutrient requirement. This protocol can be used for genetic transformation studies and for micropropagation of elite cultivars of *J. curcas*.

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