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Shoot bud proliferation from axillary nodes and leaf sections of non-toxic *Jatropha curcas* L.

M. Sujatha[1,*], H.P.S. Makkar[2] and K. Becker[2]
[1]*Crop Improvement Section, Directorate of Oilseeds Research, Rajendranagar, Hyderabad 500 030, India;* [2]*Institute for Animal Production in the Tropics and Subtropics, University of Hohenheim (480), D-70593, Stuttgart, Germany; *Author for correspondence (e-mail:mulpurisujata@yahoo.com; phone: +91-040-24015345; fax: +91-040-24017969)*

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Abstract

Protocols for *in vitro* propagation of non-toxic variety of *J. curcas* through axillary bud proliferation and direct adventitious shoot bud regeneration from leaf segments have been established. Shoot bud proliferation from axillaries was assessed on an initial basal Murashige and Skoog (MS) salt medium supplemented with different concentrations of benzyladenine (BA), kinetin and thidiazuron (TDZ) followed by subculture to medium with 4.4–8.9 μM BA. Regardless of the concentration of BA in the subculture medium, shoot multiplication rate was optimum (10–12.3) with primary culture on medium supplemented with 2.3–4.5 μM TDZ. Efficient adventitious shoot regeneration from leaf tissues was achieved with culture on medium with 8.9–44.4 μM BA + 4.9 μM indole-3-butyric acid (IBA) followed by transfer to medium supplemented with 8.9 μM BA + 2.5 μM IBA. Similarity index between toxic Indian variety and the non-toxic variety based on 435 RAPD markers was 96.3%. Crossing studies followed by phorbol ester quantitation revealed that outcrosses with toxic *J. curcas* do not affect the phorbol ester content of seeds borne on the non-toxic variety.

Abbreviations: BA – N^6-benzyladenine; IBA – indole-3-butyric acid; MS – Murashige and Skoog (1962) medium; RAPD – random amplified polymorphic DNA; TDZ – 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron)

Introduction

Jatropha curcas L. (Euphorbiaceae) – a multipurpose plant is valued not only for its medicinal properties and resistance to various stresses but also for its use as an oilseed crop (Heller 1996; Openshaw 2000). Depletion of petroleum resources is creating an opportunity for exploitation of vegetable oils as bio-diesel all over the world. Major sources of bio-diesel include rapeseed (USA), sunflower (Italy and Southern France), soybean (USA and Brazil), oil palm (Malaysia), linseed (Spain), cotton seed (Greece), beef tallow (Ireland) and *Jatropha* (Nicaragua and South America) (Jayasingh 2004). Among the potential candidate crops, *J. curcas* has attracted the interest of various developmental agencies in the tropics and subtropics due to its easy adaptability to semi arid marginal sites, use of the oil as a diesel fuel substitute and its use in erosion control. Unlike

other non-edible oils that are obtained from tree species having a long gestation period, *J. curcas* is a shrub and starts producing fruits from the second year, which stabilizes by the fourth year. High density plantation of *J. curcas* as an energy crop may provide energy on a regular basis annually for a period of 40–45 years without replanting unlike other fuel wood crops. It is a promising and profitable agro-forestry crop for poverty alleviation and generating self-employment in rural areas ensuring optimum utilization of land and manpower.

The seeds of *J. curcas* L. contain up to 60% oil with a fatty acid pattern similar to that of edible oils. The amino acid composition, the percentage of essential amino acids and the mineral content of the press cake can be compared to those of other seeds and press cakes used as fodder. The only major limitation of this crop is that the seeds are toxic and the press cake cannot be used as fodder despite having the best protein composition as per FAO standards. Due to the presence of several different toxic substances including a lectin (curcin), phorbol esters, saponins, protease inhibitors and phytates the seeds, press cake and the oil of *J. curcas* cannot be used for human or animal nutrition (Makkar et al. 1998). Increased production of *J. curcas* as a fuel source will increase the production of the toxic by-product (meal), which cannot be utilized as feed. A non-toxic variety has been found in the Papantla region of Veracruz State in Mexico, which is suitable for human consumption after roasting. The innocuous nature of this *J. curcas* variety was established using fish and rats as experimental models (Becker and Makkar 1997). Cultivation of the non-toxic *J. curcas* variety assumes utmost importance as it can provide edible oil and seed cake for livestock and gives value addition to the crop. To meet the large-scale demand and ensure easy supply of this elite material, there is a need to establish clonal seed orchards and develop mass multiplication techniques. *J. curcas* is the most primitive species of the genus and forms artificial and natural hybrid complexes readily and poses a problem to the genetic fidelity (Dehgan 1984; Prabakaran and Sujatha 1999). Macro propagation through stem cuttings is possible but the seed yields are low and the established plants are not deep rooted and hence, are easily uprooted. This is a major constraint for establishment of plants propagated through stem cuttings on poor and marginal soils. Tissue culture techniques offer rapid and continuous supply of the planting material. Evaluation of tissue culture propagated plants of *J. curcas* revealed that they were on par with seed propagated plants in terms of yield and yield related traits (Sujatha and Mukta 1996). Protocols for high frequency shoot regeneration from various explants of *J. integerrima* and *J. curcas* have been developed (Sujatha and Dhingra 1993; Sujatha and Mukta 1996; Sujatha and Reddy 2000). However, investigations on tissue culture in *J. curcas* were confined to adventitious shoot regeneration (Sujatha and Mukta 1996). The present investigation has been undertaken to develop *in vitro* propagation techniques for non-toxic *J. curcas* through axillary and adventitious shoot bud regeneration and to assess the possibility of cross contamination of non-toxicity with toxic Indian *J. curcas*.

Materials and methods

The seed material of the non-toxic variety of *J. curcas* was obtained from Dr Klaus Becker of The University of Hohenheim, Stuttgart, Germany for developing tissue culture protocols for mass propagation and carrying out studies on transmission of toxicity.

In vitro propagation

Nodal explants (3 cm) and leaves at 3rd–4th node from the apex were collected from 3-month-old plantlets. The explants were surface disinfested with 0.1% mercuric chloride (12 min for nodes; 8 min for leaves) followed by five rinses in sterile distilled water. The nodal explants were trimmed (1–1.5 cm) at the base and cultured with the cut surface in contact with the medium surface. Leaves were cut into 1.0 cm^2 segments and placed with the abaxial side in contact with the medium. Culture medium consisted of MS (Murashige and Skoog 1962) salts and vitamins, 30 g l^{-1} sucrose and 0.7% agar (Hi-media, India). For axillary bud proliferation nodal explants were cultured on medium supplemented with kinetin (2.3–46.5 μM), BA (2.2–44.4 μM) and TDZ (2.3–45.4 μM) individually. Following culture for 4 weeks, the responding explants were transferred to medium supplemented with 4.4–8.9 μM BA.

The suitability of BA–IBA medium for adventitious shoot regeneration from leaf discs of *J. curcas* and its hybrids has previously been reported (Sujatha and Mukta 1996; Prabakaran and Sujatha 1999; Sujatha and Prabakaran 2003) but the effective concentrations favouring optimal shoot regeneration varied with the genotype. The primary medium for adventitious shoot regeneration from leaf segments of non-toxic *J. curcas* was supplemented with BA (4.4–44.4 μM) and 4.9 μM IBA and the subculture medium was supplemented with 4.4 μM BA alone or 4.4–8.9 μM BA in combination with 2.5 μM IBA. Irrespective of the mode of regeneration, after the second passage the proliferating shoots were subcultured at 3-week intervals to medium supplemented with 2.2 μM BA for further multiplication and elongation. The elongated shoots were rooted on medium comprising of half strength MS basal salts and supplemented with 5.4 μM NAA. Rooted shoots were acclimatized in plastic pots through maintenance of high humidity for a week by covering with a plastic bag and then transferred to the field.

The pH of the medium was adjusted to 5.6 ± 0.1 and autoclaved at 104 kPa and 120 °C for 20 min. The cultures were incubated at 26 ± 2 °C under a 16 h photoperiod using cool, white fluorescent lights (30 $\mu mol\ m^{-2}\ s^{-1}$). For experiments on axillary bud proliferation, each treatment consisted of three replicates of 20 culture tubes (150×25 mm) containing 15 ml medium with one nodal explant. Observations on number of sprouted buds were recorded after 4 and 2 weeks of culture on the primary and subculture medium, respectively. For experiments on induction of adventitious shoots from leaf tissue, each treatment consisted of three replications with 20 culture tubes having three leaf segments in each and observations on the number of leaf discs showing shoot differentiation were recorded on primary and subculture media after 4 and 2 weeks of culture, respectively. Data were subjected to statistical analysis and the means were separated according to DMRT test at $\alpha = 0.05$.

Molecular analysis

For RAPD analysis, DNA was isolated from young, fully expanded leaves using the method of Dellaporta et al. (1983). Following initial screening with 10 primer sets RAPD profiles were generated using 120 random decamer primers from six sets (OPD, OPG, OPJ, OPK, OPL and OPM from Operon Technologies, Alameda, USA) individually in polymerase chain reactions (PCRs) following the protocol of Williams et al. (1990). The PCR mixture (20 μl) contained 1.2 U *Taq* DNA polymerase, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 100 μM of each dNTP, 2 μl of the primer at a final concentration of 0.25 μM and 50 ng of template DNA. Amplification products were separated by electrophoresis on a 1.8% agarose gel (Genei, Bangalore, India) in 1× TAE buffer (0.04 M Tris–acetate, 0.01 M EDTA) for 1 h 20 min at 75 V and visualized by staining with ethidium bromide. Similarity index was calculated as percentage of similar pairs to the total number of visually scorable band pairs.

Determination of toxicity

For determination of phorbol ester content, plants of both genotypes were selfed, sib-mated and crossed. At flowering, the female flowers of the ovule parents were dusted with pollen from the male parent and crosses were effected in both direct and reciprocal directions. The seeds from these crosses were harvested at maturity. Phorbol esters were determined as mg TPA (equivalent to phorbol-12-myristate 13-acetate) in 1 g shelled kernels according to Becker and Makkar (1998).

Results and discussion

Axillary bud proliferation

Culture of nodal explants on medium supplemented with a range of concentrations of the three cytokinins resulted in differential response. Regardless of the cytokinin, in majority of the cases only one to three shoot buds sprouted on the primary medium in 1 month of culture period (Table 1). Nodes cultured on medium with kinetin showed no visible signs of explant differentiation except for bud emergence with very low axillary proliferation and elongation of the existing bud into a single shoot. However, in case of medium

Table 1. Effect of cytokinins on the number of shoots per nodal explant of non-toxic *J. curcas*.

Cytokinin	Concentration (μM)	Primary culture	Subculture medium	
			4.4 μM BA	8.9 μM BA
Kinetin	2.3	0^{d}	0^{f}	0.3 ± 0.5^{d}
	4.7	1.0 ± 0^{c}	0.6 ± 0.5^{ef}	2.3 ± 1.2^{bcd}
	9.3	1.0 ± 0^{c}	1.7 ± 0.5^{bcdef}	3.0 ± 0^{bcd}
	23.2	3.3 ± 0.5^{a}	1.3 ± 0.5^{cdef}	1.3 ± 0.5^{cd}
	46.5	1.3 ± 0.5^{bc}	1.0 ± 0^{def}	1.0 ± 0^{cd}
BA	2.2	2.0 ± 0^{b}	3.7 ± 0.5^{ab}	4.0 ± 0.8^{bc}
	4.4	2.0 ± 0^{b}	3.3 ± 0.9^{abc}	4.3 ± 0.5^{bc}
	8.9	2.0 ± 0.8^{b}	4.0 ± 0.8^{a}	5.0 ± 0.8^{b}
	22.2	2.2 ± 0.6^{b}	2.7 ± 0.5^{abcde}	3.7 ± 1.7^{bcd}
	44.4	2.0 ± 0.8^{b}	2.3 ± 0.5^{abcde}	2.0 ± 0.8^{bcd}
TDZ	2.3	1.0 ± 0^{c}	3.7 ± 1.7^{ab}	10.0 ± 4.2^{a}
	4.5	1.0 ± 0^{c}	3.0 ± 2.8^{abcd}	12.3 ± 1.7^{a}
	9.1	1.0 ± 0^{c}	1.3 ± 0.5^{cdef}	2.7 ± 0.9^{bcd}
	22.7	1.0 ± 0^{c}	1.0 ± 0^{def}	2.0 ± 0.8^{bcd}
	45.4	1.0 ± 0^{c}	0.6 ± 0.5^{ef}	1.3 ± 0.5^{cd}

Means in a column followed by same letters are not significantly different according to DMRT at $\alpha = 0.05$.
Number of observations used to calculate the means = 60 (20 nodal explants/replication and three replications).
Data scored after 4 and 2 weeks of culture in primary and subculture media, respectively.

supplemented with 23.2 μM kinetin, the number of shoots per explant varied from 2 to 4 with an average of 3.3 shoots per nodal explant. The length of the shoot increased with increasing concentration of kinetin. Medium supplemented with BA resulted in development of greenish white callus at the base with well-differentiated shoots from the axillary bud. The length of the shoot decreased with increasing concentration of BA. Incorporation of TDZ favoured excessive expansion of the base of the nodal explant and the node assumed a flattened appearance.

Significant differences were observed on subculture of nodal explants cultured on different cytokinins to 4.4–8.9 μM BA. On medium supplemented with 4.4 μM BA, shoot multiplication was observed only in explants transferred from medium with TDZ and BA while those from kinetin failed to proliferate. Number of shoots per explant was maximum (4.0) in nodes transferred from 4.4 μM BA.

Nodal explants showed positive response on transfer to medium with 8.9 μM BA and multiplication rate was higher as compared to those transferred to 4.4 μM BA. Maximum shoot proliferation rate (10–12.3) was obtained with nodes cultured initially on medium with 2.3–4.5 μM TDZ. Nodal explants transferred from medium supplemented with BA and kinetin produced shoots from axillaries while those from TDZ supplemented media differentiated shoots directly without any callus intervention from the flattened base. Nodes subcultured from TDZ supplemented media continued to expand and produced new shoots up to third subculture indicating its carry over effect. Culture of axillary buds for 12 weeks involving 4 subcultures resulted in production of 4, 10 and 24 shoots per node from explants initially cultured on medium with kinetin, BA and TDZ, respectively.

Reports on adventitious shoot regeneration for *J. curcas* are available (Sujatha and Mukta 1996) and this constitutes the first report of axillary bud proliferation for mass propagation of this species. In most of the *Euphorbiaceae* members, presence of cytokinin alone gave optimal shoot proliferation such as 2-ip in *Euphorbia lathyrus* (Lee et al. 1982), and BA in *E. lathyrus* (Tideman and Hawker 1982; Ripley and Preece 1986), *E. peplus* and *E. tannensis* (Tideman and Hawker 1982) and cassava (Nair et al. 1979). In the recent past, TDZ has become an integral component in tissue culture of woody as well as herbaceous crop species (Huetteman and Preece 1993). The favourable influence of TDZ on shoot proliferation from meristematic explants of castor was reported (Sujatha and Reddy 1998). In case of castor, TDZ had a stimulatory effect in

promoting shoot proliferation from embryo explants while BA favoured maximum shoot regeneration from axillary buds. However, in case of *J. curcas*, TDZ promoted higher shoot regeneration frequency from axillaries as compared to BA. The differential response of these two systems to the same cytokinin may be attributed to the correlative interaction between the type of tissue and the concentration of endogenous growth regulators (George 1993).

Adventitious shoot regeneration

Owing to the limitation of axillary buds, direct adventitious shoot regeneration using leaf explants was attempted. Leaf explants enlarged and developed greenish white or white callus at the cut margins. Adventitious buds originated directly from the region close to the induced callus. Buds (3–11 per explant) were observed in clusters within 3 weeks of culture initiation. On primary medium maximum frequency of shoot bud regeneration was recorded on medium supplemented with 22.2 μM BA in conjunction with 4.9 μM IBA (Table 2). Subculture of the leaf explants to medium supplemented with 2.2 μM BA or 4.4–8.9 μM BA with 2.5 μM IBA led to further differentiation of shoots. Of the three combinations tested, medium supplemented with BA and IBA gave a higher frequency of shoot regeneration. Maximum regeneration (80–90%) was obtained with leaf explants cultured initially on medium with 8.9–44.4 μM BA in combination with 4.9 μM IBA and transferred to medium with 8.9 μM BA and 2.5 μM IBA. Independent of the frequency of shoot regeneration on primary medium, leaf explants cultured on medium with 8.9 μM BA + 4.9 μM IBA gave highest frequency of regeneration on all the media tested for subculture.

Leaf discs from *J. tanjorensis*, a natural interspecific hybrid elicited maximum caulogenic response on medium with 4.4 μM BA + 2.5 μM IBA (Prabakaran and Sujatha 1999). However, in the present investigation maximum shoot regeneration was recorded on medium with 8.9 μM BA + 2.5 μM IBA. Shoot regeneration frequencies obtained for non-toxic *J. curcas* (80–90%) are much higher than those reported for Indian *J. curcas* – (50%; Sujatha and Mukta 1996), *J. tanjorensis* – involving *J. curcas* as a putative parent – (69–75%; Prabakaran and Sujatha 1999) and the ornamental hybrids between *J. curcas* and *J. integerrima* (71%; Sujatha and Prabakaran 2003). Differences among genotypes for direct organogenesis from leaf tissues may be due to innate differences in the concentration of endogenous hormones.

Molecular analysis

Of the 120 primers tested, amplification was observed in both the samples with 95 primers. The number of bands per primer varied between one and 13. Polymorphism was generated with 14 primers and maximum polymorphism was generated with primers from OPJ and OPM sets while no polymorphism was detected with primers from OPL set (Figure 1a and b). Similarity index between the two genotypes based on 435 bands scored was 96.3%. The polymorphism generated with these primers serves as reference fingerprints for distinguishing the non-toxic variety from the toxic Indian cultivar.

Table 2. Influence of BA and IBA on adventitious shoot regeneration (%) from leaf segments of non-toxic *J. curcas*.

Concentration of BA (μM) + 4.9 μM IBA	Primary medium	Subculture medium		
		2.2 μM BA	4.4 μM BA + 2.5 μM IBA	8.9 μM BA + 2.5 μM IBA
4.4	46^{b}	8^{bc}	18^{b}	34^{b}
8.9	54^{ab}	29^{a}	76^{a}	90^{a}
22.2	79^{a}	12^{b}	69^{ab}	83^{a}
44.4	40^{b}	3^{c}	60^{ab}	80^{a}
CV (%)	18	35	15	19

Means in each column followed by same letters are not significantly different according to DMRT at $\alpha = 0.05$.
Number of observations used to calculate the means = 180 (60 leaf segments/replication and three replications).
Data scored after 4 and 2 weeks of culture in primary and subculture media, respectively.

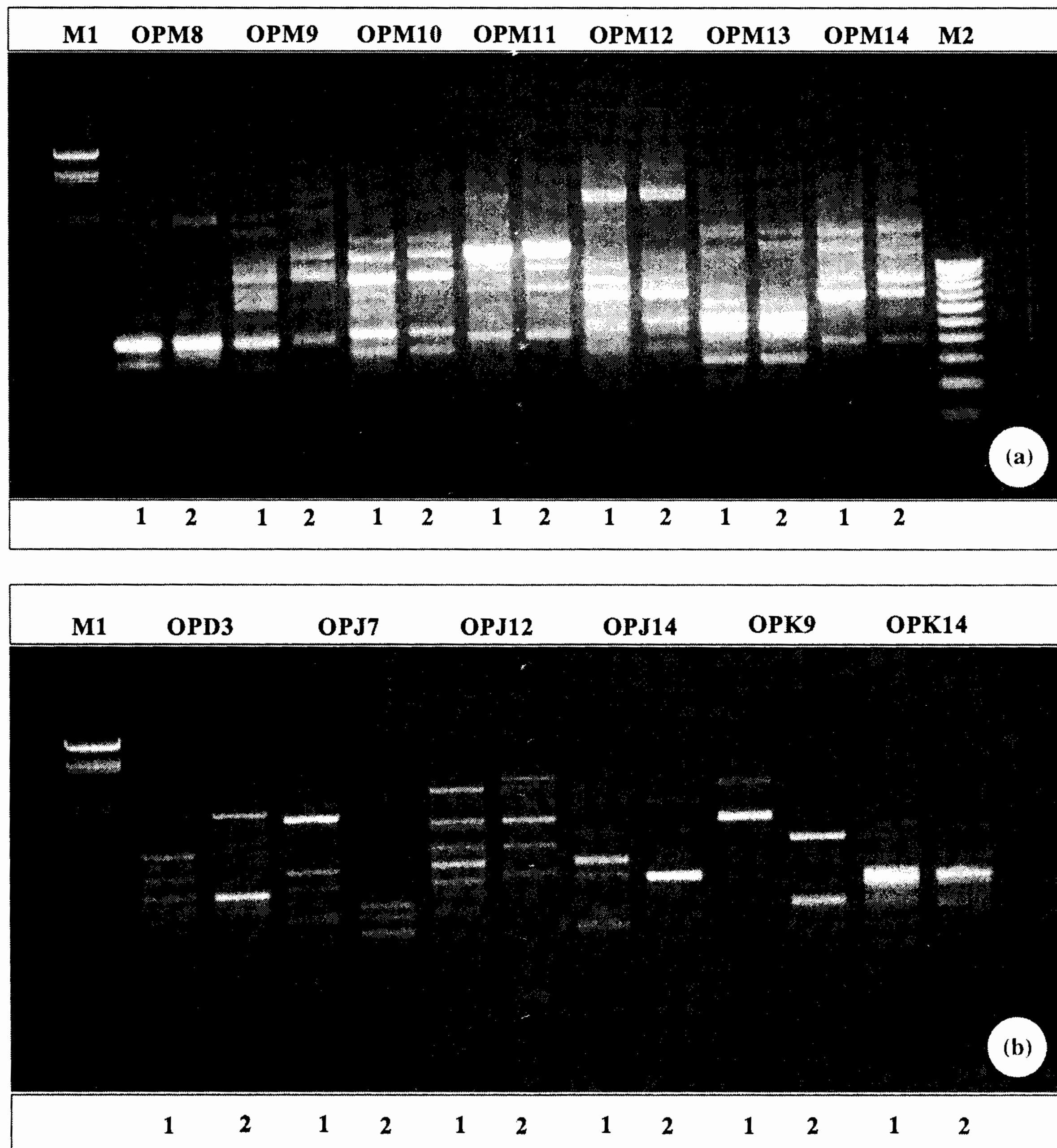

Figure 1. RAPD analysis of toxic Indian variety and the non-toxic variety of *J. curcas*. (a) PCR amplification with OPM 8–14. 1 and 2 represent toxic Indian and non-toxic varieties, respectively. Lanes designated as M1 represents λDNA double digest with *ECoRI* and *Hind*III restriction enzymes, M2 is a 100 bp ladder. (b) PCR amplification with six RAPD primers showing polymorphism. 1 and 2 represent toxic Indian and non-toxic varieties, respectively. Lane designated as M1 represents λDNA double digest with *ECoRI* and *Hind*III restriction enzymes.

Determination of toxicity

Crosses were effected between the two varieties in both directions to check for the effect of pollen from toxic *J. curcas* on the phorbol ester content of seeds of non-toxic variety and vice versa. The range of phorbol ester content in the toxic strains was around 20–60 times greater than the range in

Table 3. Phorbol ester contents in different *Jatropha* varieties and crosses involving toxic and non-toxic varieties.

Material	mg of TPA in 1 g shelled kernels
Toxic Cabo verde *J. curcas*	1.30
Toxic Indian *J. curcas*	2.21
Non-toxic Mexico (original seed)	0.06
Non-toxic Mexico (After 1 growing season in India)	0.07
Indian *J. curcas* (open pollinated)	1.17
Non-toxic Mexico (open pollinated)	0.02
Indian *J. curcas* × non-toxic Mexico	1.68
Non-toxic Mexico × Indian *J. curcas*	0.05

Estimation of phorbol esters was done on F_1 seeds that were obtained from the parental plants following crossing.

the non-toxic strains. Estimation of phorbol ester contents of the F_1 seeds revealed reciprocal differences, which indicated that the phorbol ester contents are not affected by the pollen source (Table 3).

Introduction of this non-toxic variety of *J. curcas* will enhance the value of this crop in human and animal nutrition. The variety can be propagated through tissue culture and the crossing studies have clearly indicated that accidental outcrossing with toxic *J. curcas* will not affect the phorbol ester content of seeds borne on the non-toxic variety. Mass propagation through shoot proliferation from axillary and apical bud cultures has been optimized. It is a crop with low capital investment, short juvenile period and long productive periods with unlimited employment potential in rural areas (Jones and Miller 1991; Gubitz et al. 1999). Owing to its economic importance, any improvement will have far-reaching and positive implications in this neglected and under-exploited species.

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