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Somatic embryogenesis in *Jatropha curcus* L. using cotyledonary leaves

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An efficient protocol has been developed for high frequency plant regeneration through somatic embryogenesis in the biodiesel plant, *Jatropha curcus*. Cotyledonary leaves were used for the development of the embryogenic callus on MS medium with 0.2 mg/L indole-3-acetic acid (IAA), which developed within 3-4 wk. Later, somatic embryos developed on MS medium with IAA (0.2 mg/L) along with 6-benzyl aminopurine (BAP; 1.5 mg/L). Fist, globular embryo clumps were seen within 3-4 wk and then they got transformed into heart, torpedo and cotyledonary shaped embryos on the same medium after 6 wk. On further subculture on the same medium, embryos germinated with shoot and root primordia, which ultimately gave rise to complete plantlets within 4-5 wk. The pantlets were hardened and transferred to the soil. Around 50% plantlets survived in the soil.

Keywords: Cotyledonary shape, embryo, embryogenic callus, globular, heart shape, *Jatropha curcus*, torpedo shape

Jatropha curcus L. is an important biodiesel plant and a renewable substitute for fossil fuels[1]. It has potential to be grown as a renewable energy crop, which could become a viable option in the future. Besides having potential of biodiesel, Jatropha has immense qualities. Its bark has a dark blue dye, which is used for colouring clothes and fishing nets. Fruit hull and oil cake of Jatropha are rich in nitrogen, phosphorous and potassium and can be valuably used as organic manure, mulch or compost[2]. Fresh cake and oil of Jatropha also have immense insecticidal and molluscicidal properties[3]. The ether extract of the plant shows antibiotic activity against *Staphylococcus aureus* and *Escherichia coli*[4]. Jatropha oil is an environmentally safe, cost effective and renewable source of non-conventional energy as a promising substitute to diesel, kerosene, LPG, coal and firewood, etc[5]. The capability of Jatropha for growing with or without irrigation makes it promising and profitable agro-forestry crop both under rain fed and irrigated conditions ensuring optional utilization of land, man power, water and financial resources. Roots are also used for medicines[6]. Thus, Jatropha attracts a lot of interest, elicited large investments and require rapid expansion of its cultivation.

Despite of its multifarious potentialities, the plant has some regeneration constraints while propagated through seeds or through stem cuttings. There were difficulty in seed germination due to dependence on season, rainfall, time and depth of seed sowing, and having very hard seed coat, while the plants propagated through stem cuttings were uprooted easily[7]. The plant is recalcitrant in tissue culture because of the presence of latex[8]. Although, *J. curcus* has been micropropagated by different means earlier, *viz*., organogenesis through callus culture[9], somatic embryogenesis[10], direct shoot organogenesis from leaf, cotyledonary leaf and hypocotyl explants[11-13], the multiplication rate of shoots was comparatively slow, inefficient and non-repeatable. Therefore, an efficient and repeatable protocol for *in vitro* multiplication of *J. curcus* is needed to meet the increasing demand and supply of elite materials of this important plant species. Authors report here the high frequency plant regeneration of *J. curcus* through somatic embryogenesis.

Seeds of *J. curcus* were taken from 1 to 2-yr-old shrubs growing in the Department of Botany, University of Rajasthan, Jaipur campus. These seeds were soaked in tap water for 3 d (72 h), followed by drying in sunlight for about 1-2 h. The seeds were surface sterilized with 0.2% Teepol solution (a mild detergent, Central Drug House, India) for 5-8 min and rinsed at least thrice with sterile distilled water. Then, they were treated with 0.1% aqueous solution of $HgCl_2$ for 3-5 min, followed by rinsing with sterile distilled water to remove the traces of mercuric chloride. These sterilized seeds were inoculated under complete aseptic conditions on sterilized paper bridges containing only distilled water. After 10-12 d of seed germination, cotyledonary leaves, leaf and hypocotyl explants were procured from aseptically

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grown seedlings and cultured on MS medium[14] with different concentrations and combinations of auxins and cytokinins for somatic embryogenesis (Table 1). All the cultures were maintained at 26±2°C under fluorescent light (2000 lux) and 50-55% relative humidity.

Previous studies have shown that plant growth regulators, especially auxins, play a pivotal role in the development of embryogenic callus, induction of somatic embryos and their further development and culmination into plantlets. In the present study, embryogenic callus was developed from the cotyledonary leaves, when 1 cm^2 sized 4-5 leaf segments were placed on MS medium fortified with 3% sucrose and 0.2 mg/L indole-3-acetic acid (IAA). Initially, curling of the leaf piece occurred and it finally turned into embryogenic callus. Embryogenic clumps were separated carefully from the normal callus cells and regularly sub-cultured on every 21 d on the fresh MS medium supplemented with 0.2 mg/L IAA and 1.5 mg/L 6-benzyl aminopurine (BAP). As a result, there was considerable increase in the embryogenic calli and further growth into sequential development of somatic embryos, *i.e.*, globular, torpedo, heart and cotyledonary shaped embryo. In the present study, cotyledonary leaves curled after 7 d of incubation and embryogenic callus formation was observed within 25-28 d on MS medium supplemented with 0.2 mg/L IAA (Figs 1A & B). After 1 wk of subculture, the embryogenic callus differentiated into globular embryos, which converted into heart and torpedo shape and ultimately matured into cotyledonary stage embryos. At a given time, all

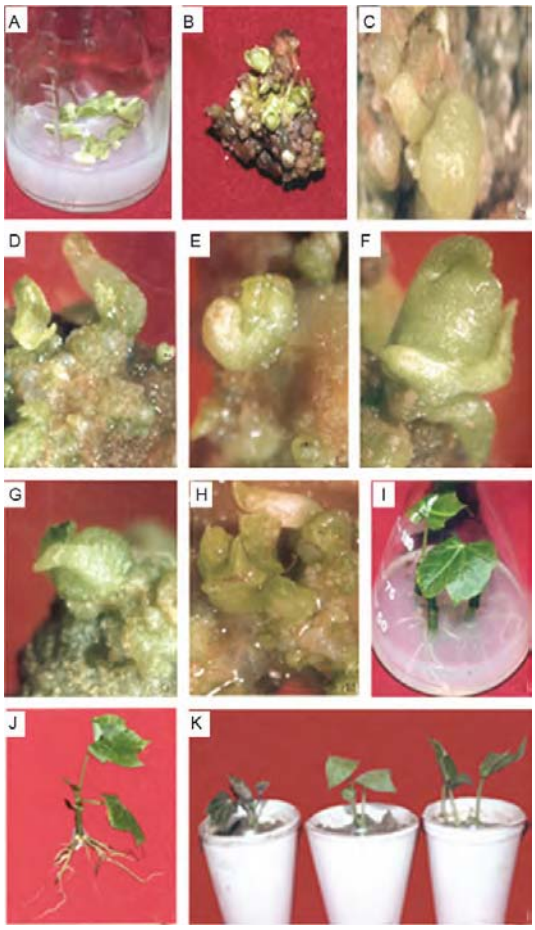

Fig. 1 (A-K)—Micropropagation of *J. curcas* plants through somatic embryogenesis: (A) Curling and callusing of cotyledonary leaf; (B) Nodulated callus on the same medium; (C) Globular embryo; (D) Heart shaped embryo; (E) Torpedo shaped embryo; (F) Cotyledonary shaped embryo; (G) Germinating embryo; (H) Development of shoot system with prominent leaves; (I) Induction of *in vitro* roots; (J) Exposed view of the complete plantlet; & (K) Hardened plantlets in the natural environment.

Table 1—Effect of plant growth hormones on callus induction from cotyledonary leaves of *J. curcus* on MS medium for somatic embryogenesis

Conc. of hormone (mg/L)	2,4-D		IAA		IBA		IAA+BAP		
	Callus initiation (d)	% response (±SD)	Callus initiation (d)	% response (±SD)	Callus initiation (d)	% response (±SD)	Conc. of IAA+BAP (mg/L)	Callus initiation (d)	% response (±SD)
0.1	15	25.3 ± 2.5	21	62.6 ± 2.1	12	15.0 ± 1.4	0.2+0.5	25	34.9 ± 2.2
0.2	15	29.0 ± 2.6	25	84.6 ± 1.5	15	20.0 ± 1.0	0.2+1.0	25	43.3 ± 1.0
0.5	20	31.0 ± 1.0	25	80.3 ± 1.5	20	35.0 ± 1.1	0.2+1.5	30	48.6 ± 2.5
1.0	20	19.6 ± 1.5	28	64.3 ± 3.0	20	28.0 ± 2.1	0.2+2.0	30	40.4 ± 1.1
1.5	25	17.0 ± 2.0	28	50.0 ± 1.0	28	26.0 ± 1.0	0.2+2.5	30	34.2 ± 2.1
2.0	25	14.6 ± 1.5	32	49.6 ± 1.5	30	25.0 ±1.0	0.2+3.0	32	31.7 ± 1.0
2.5	Nil	Nil	30	44.6 ± 2.5	32	24.6 ± 1.5	Nil	Nil	Nil
3.0	Nil	Nil	30	38.3 ± 3.7	32	20.0 ± 1.0	Nil	Nil	Nil

In columns, the values with the same letter are not significantly different at 5% level of significance by ANOVA test.

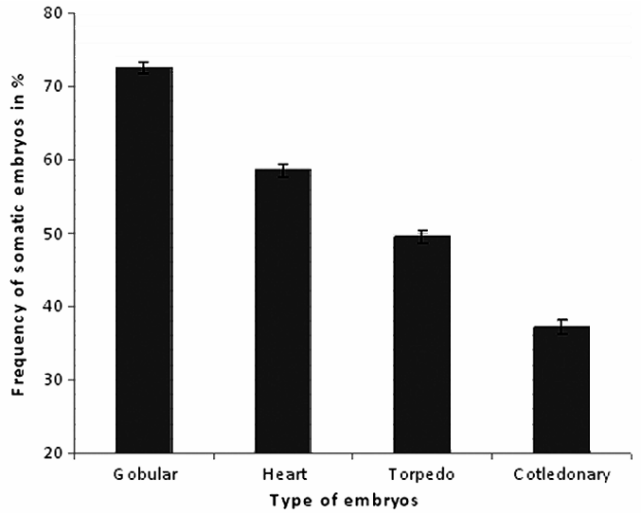

Fig. 2—Frequency of different stages of somatic embryos on MS medium with IAA 0.2 mg/L along with BAP 1.5 mg/L after 45 d

stages of embryo development could be seen in the same clump of cells. Thus, induction of somatic embryogenesis and their sequential development with varied percentage of frequency was attained on MS medium supplemented with IAA 0.2 mg/L (Figs 1C-F), whereas maturation and germination was completed on 0.2 mg/L IAA along with 1.5 mg/L BAP (Figs 1G, H & 2). The conversion of globular embryos subsequently into cotyledonary embryos took about 40-45 d. Similar types of results have been reported in many crops[15-18]. Further, germinating embryos showed shoot and root initiation leading to maturation of plantlets on the same medium within next 18 d (Fig. 1I). Nagesh *et al*[18] have also reported that shoots initiated from the somatic embryos did not require plant hormone for *in vitro* root induction. However, shoots initiated from the somatic embryos required IBA for root initiation in the studies of Sharma *et al*[19].

In vitro developed plantlets with well developed shoots and roots were exposed (Fig. 1J) and transferred to the thermocol cups having soilrite (Fig. 1K) for acclimatization. High humidity was maintained by covering the pots with inverted glass beakers. After 30 d, plants were found well established and growing. They were then transferred to the natural environment and about 50% rate of survival was recorded.

In conclusion, a well defined simple, efficient and reliable protocol has been developed for an efficient somatic embryo production in a recalcitrant plant like *J curcus*, which can be further used in the transformation studies. *In vitro* plant regeneration *via* somatic embryogenesis and organogenesis is essential for conservation of plant genetic resource management.

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