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PLANT REGENERATION FROM LEAF AND PETIOLE EXPLANTS OF *JATROPHA CURCAS* L.: A BIOFUEL PLANT

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ABSTRACT
The use of *Jatropha curcas* plants for large-scale bio-diesel production is of great interest with regard to solving the energy shortage, reducing the carbon emission and increasing the income of farmers. To exploit it to the maximum extent, one method of plant reproduction and biological active component formation is the use of tissue culture. To date, the studies of *J. curcas* tissue culture have been confined to leaflet, petiole, cotyledon, hypocotyl and axillary bud. However, the regeneration frequencies, especially adventitious bud inductive frequencies, were still low. Hence protocols for *in vitro* propagation of *J. curcas* through leaf and petiole explants with high frequency (85 %) have been established. The leaf and petiole explants produced higher percentage of calli on Murashige and Skoog (MS) medium supplemented with 0.5 mg/l benzyladenine (BA) and 1.0 mg/l 2,4 dichloro phenoxy aceticacid (2,4 D) after four weeks. Efficient shoot regeneration obtained from MS medium supplemented 1.5 mg/l BA, 0.5 mg/l IBA and 1.5 mg/l GA_3 for leaf and petiole derived calli. Finally, the shoots were rooted on ½ MS media supplemented with 0.3 mg/l IBA. Regenerated plants with well developed shoots and roots were successfully transferred to green house, without visible detectable variation.

KEYWORDS: *in vitro* propagation; Hypocotyl; Cotyledonary leaf; Callus; Shoot regeneration, regeneration frequency, benzyladenine (BA), 2,4 dichloro phenoxy aceticacid (2,4 D)

INTRODUCTION

Depletion of petroleum resources is creating an opportunity for exploitation of edible and non edible oils as bio-diesel all over the world (Sujatha *et al*., 2005). Biodiesel is less harmful to the environment in that its production and combustion reduces emission of greenhouse gases by 41 % relative to fossil fuel and emits less particulate matter than diesel upon combustion (Hill *et al*., 2006; Yuan *et al*., 2008). To avoid competition against food oil, non-edible oil such as *Jatropha* oil is preferable and promising for large-scale biodiesel production. Originating in the Caribbean, *Jatropha* was spread as a valuable hedge plant to Africa and Asia by Portuguese traders. The name is derived from Greek words —iatros meaning physician and trophe meaning nutrition. The *Jatropha* plant has several utilities as its seeds are used against constipation, the white latex serving as a disinfectant for mouth infection; it also stops bleeding, the leaves are used against malaria, grown as a live fence and used as an alternate commercial crop, prevents soil erosion, production of anti–cancerous agent, soap making, seed cake is rich in nitrogen and phosphorus, used as manure and vanillin production (Das and Velkataiah 2000; Jha *et al*., 1992; Kumar and Sharma 2008; Rajesh *et al*., 2007;). Ever-since it was established that *Jatropha* methyl ester is biodiesel of an exceptional quality, there has been surge of interest in *Jatropha* across the globe for its cultivation. In case of *Jatropha*, conventional propagation through seeds is not reliable due to poor germination rate and vegetative cuttings show delayed rooting which hinder to meet the demand of large scale quality planting material (Openshaw, 2000). To exploit it to the maximum extent, one method of plant reproduction and biological active component formation is the use of tissue culture which provides an alternative approach to the plants which are difficult to cultivate or has a long cultivation period, or has a low yield. Use of tissue culture techniques helps for rapid production of *Jatropha* to meet large scale demand and ensure easy supply of the elite plant material as well as germplasm conservation. In recent years, plant regeneration in *J. curcas* has been accomplished through direct and indirect organogenesis from various explants, including petiole and hypocotyls (Sujatha amd Mukta 1996), mature leaf (Deore and Johnson 2008), auxiliary node (Shrivastva and Banerjee 2008; Sujatha *et al*., 2005), Stem (Singh *et al*., 2010) and via somatic embryogenesis from mature leaf explants (Jha *et al*., 2007). However, the regeneration frequencies, especially adventitious bud inductive frequencies, were still low. To find better explant resources for regenerating plants, leaf and petiole were tested. The objective of this work was to develop efficient regeneration system from, leaf and petiole of *Jatropha curcas* L.

MATERIALS & METHODS

Ex vivo leaf explants and petioles from one year old field grown *Jatropha* plants were collected, surface sterilized with 0.1% $HgCl_2$ for 10 min followed by 0.1% $CuSO_4$ treatment for 3 min and rinsed with sterile water four times before inoculation. All media were adjusted to pH

5.8 with 1 N KOH or 1 N HCl, solidified with 0.7% (w/v) agar, and autoclaved at 121°C for 20 minutes. The cultures were incubated at 25°C ± 2°C in darkness or under 14 h photoperiod using cool, white fluorescent lights (during subculture).

Different explants (leaf and petiole) were cultured on MS medium supplemented with BAP in combination with IBA, 2, 4-D, NAA to optimize callus induction (Table 1). The explants were maintained on callus induction medium for 4 weeks by sub-culturing at 7 days interval. Callus induction frequency was calculated using the formula (Frequency of callus induction = Explants induced callus/number of explants cultured X 100). TDZ treatment was given to calli derived from leaves and petioles for 4 days. After TDZ treatment, calli were transferred on to MS medium supplemented with BAP in combination with IBA and GA_3 (Table 1) to find out an optimum combination of growth hormones for shoot induction and regeneration.

Elongated shoots with three to four leaves were excised and cultured on MS medium supplemented with different concentrations of IBA and NAA (Table 1). The percentage of rooting was recorded after 3 weeks. Rooted shoots were carefully taken out of the medium and washed thoroughly in sterilized distilled water to remove the medium attached to roots. The plants were transferred to sterilized potting mixture (sand and silt at 1:1) for hardening.

TABLE 1 : Different medium used for callus induction, shoot regeneration and rooting

Callus induction medium						
BAP (mg/l)	BAP (mg/l)	IBA (mg/l)	GA_3 (mg/l)	Shoot regeneration medium		
0.5	1.5	0.05	0.5	BAP (mg/l)	IBA (mg/l)	GA_3 (mg/l)
0.5	1.5	0.1	0.25	1.5	0.05	0.5
1.0	1.5	0.5	1.5	1.5	0.1	0.25
1.0	0.1	0.1	0.1	1.5	0.5	1.5
1.5	0.25	0.1	-	0.1	0.1	0.1
1.5	0.25	-	-	0.25	0.1	-
-	-	0.5		0.25	-	-
1.0		0.5				
0.5		1.0				
1.0		2.0		Rooting medium		
1.0			0.5	IBA (mg/l)	NAA (mg/l)	
2.0			0.5	0.3	-	
				0.5	-	
				-	0.3	
				-	0.5	

RESULTS

Callus cultures were initiated from leaves and petioles on MS media supplemented with different levels and combinations of growth regulators *viz*., BAP, IBA, 2, 4-D and NAA. A significant difference in callus induction was observed among different explants and media composition. Among two explants studied, petiole was found to show highest frequency of callus induction. Besides, they tend to initiate callus earlier than leaves. The callus appeared in petiole explants 8 days after culture, while it was 10 days in leaves. Of different levels of BAP and NAA tested, the medium containing BAP (1 mg/l) + NAA (0.5 mg/l) showed the highest callus induction frequency of 89 % in leaf explants. Among the different combinations of BAP and 2, 4-D, the combination of BAP at 0.5 mg/l and 2, 4-D at 1 mg/l was found to exhibit a highest callus induction frequency of 89.40% in petiole explants. The combination of BAP at 1 mg/l and IBA at 0.5 mg/l produced callus at a frequency of 87. 00 %. Of different levels of BAP and 2,4-D tested, the medium containing BAP 0.5 mg/l + 2,4-D at 1 mg/l showed the highest callus induction frequency of 89.40 % in petiole explants (Fig. 1). Treatment of calli in MS medium containing TDZ 0.3 mg/l for 4 days showed shoot bud initiation after 2 wk on MS medium supplemented with BAP 1 mg/l + IBA 0.5 mg/l + GA_3 1.5 mg/l (Fig. 4c). The response of calli derived from leaves and petioles to regeneration on MS media supplemented with different levels and combinations of BAP, IBA and GA3 are calculated. Regeneration efficiency at 70 % and 60 % with single shoot regeneration from leaf and petiole calli respectively was observed on MS medium supplemented with BAP 1.5 mg/l + IBA 0.05 mg/l + GA_3 0.5 mg/l (Fig. 2; Fig. 4c). Shoot induced from calli which were grown on MS medium supplemented with BAP 1.5 mg/l + IBA 0.5 mg/l + GA_3 1.5 mg/l gave rise to multiple shoots with 85 % and 70% regeneration efficiency for leaf and petiole calli respectively (Fig. 4d; Fig. 2). The shoots of height of 2-3 cm obtained on MS medium fortified with BAP 0.3 mg/l + GA_3 0.3 mg/l concentration after 3 wk (Fig. 4e).

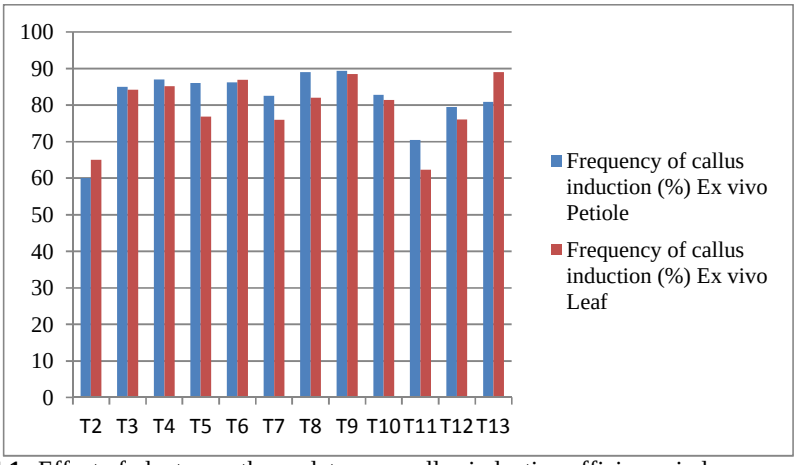


FIGURE 1:-Effect of plant growth regulators on callus induction efficiency in leaves and petioles

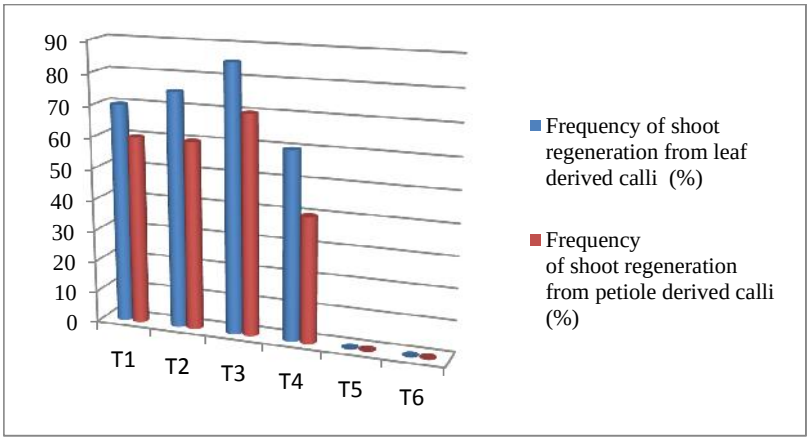


FIGURE 2:-Effect of plant growth regulators on regeneration efficiency in leaf derived calli and petiole derived calli

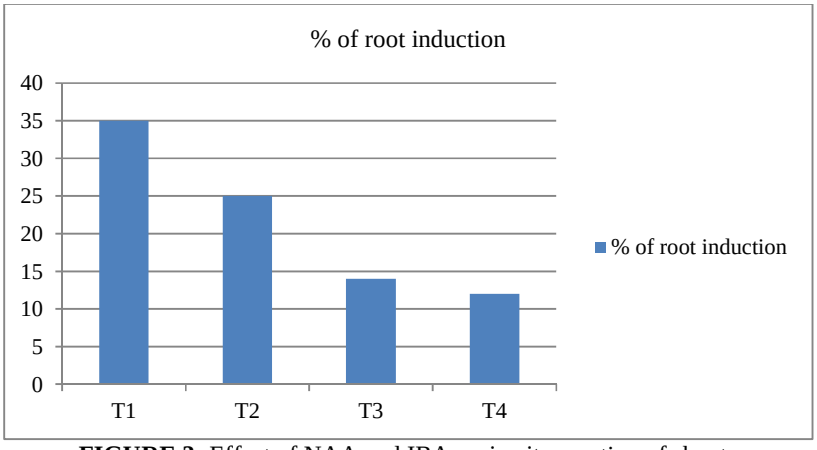
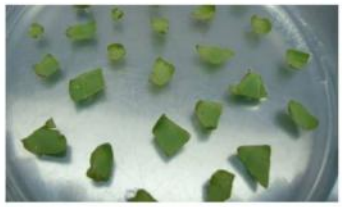
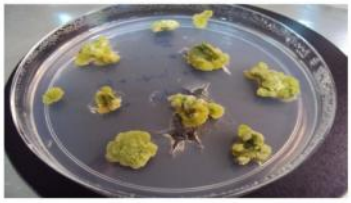


FIGURE 3: Effect of NAA and IBA on *in vitro* rooting of shoots

Individual shoots were excised and successfully rooted on MS medium supplemented with 0.3 mg/l IBA. A higher rate of root induction (35 %) was obtained after 3 wk on MS medium + IBA 0.3 mg/l (Fig.4f; Fig. 3). After proper rooting for 3 wk plantlets were taken out, washed in running tap water to remove agar and were placed for hardening. Rooted plantlets were transferred to plastic cups filled with soil mixture (sand and silt at 1:1) covered with transparent plastic bags, maintained at a temperature of 25 ± 2 °C for 10 days. These plants were maintained at room temperature for 1 week before they were transferred to greenhouse. The tissue culture protocol optimized in this study took 16 weeks to produce complete plants in *in vitro* from leaf and petiol explants.

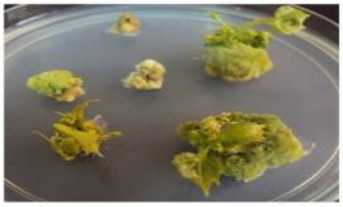


a. Leaves inoculated on callus induction medium (MS + BAP 1 mg/l + NAA 0.5 mg/l)



b. Calli obtained from leaves after 4 weeks on (MS + BAP 1 mg/l + NAA 0.5 mg/l)

c. Shoot initiation from calli on shoot regeneration medium (MS + BAP 1.5 mg/l+ IBA 0.5 mg/l + GA_3 1.5 mg/l)



d. Shoot multiplication after 3 weeks incubation in shoot regenration medium (MS + BAP 1.5 mg/l+ IBA 0.5 mg/l + GA_3 1.5 mg/l)

e. Elongation of regenerated shoots on elongation medium (MS + BAP 0.3 mg/l + GA_3 0.3 mg/l)

f. Rooting of elongated shoot on MS + IBA 0.3 mg/l

g. *In vitro* regenerated plant transferred to soil

FIGURE 4: In vitro *vitro* regeneration of *Jatropha curcas* L.

DISCUSSION

Jatropha is an ideal plant for using wasteland and its non-edible oil is a prime choice for biodiesel production. It also has problems of poor seed viability, low germination, scanty and delayed rooting in seedlings and vegetative cuttings (Deore and Johnson, 2008; Kaewpoo and Te-chato, 2009). Because of these problems related with conventional propagation, *Jatropha* improvement programme by modern methods of agro biotechnology are of interest worldwide (Das and Velkataiah, 2000). In the present study, petioles and leaves were used as explants for the establishment of cultures of *J. curcas*. Callus induction efficiency of petiole and leaf explants was found to vary on MS media supplemented with different plant growth regulators. Micropropagation of *J. curcas* has been reported by various authors using explants such as hypocotyls, mature leaf, stem and auxiliary node (Hill *et al*., 2006; Kalimutuhu *et al*., 2007; Kumar and Sharma 2008; Kumar *et al*., 2010). The morphology of calli observed was dark green to pale yellow color and compact to fragile. The calli developed from MS medium with BAP (1 mg/l) and NAA (0.5 mg/l) was found to be green fragile with 89% calli induction efficiency. Similar type of calli morphology was reported with leaf explants cultured on MS medium supplemented with BAP 0.5 mg/l + NAA 1 mg/l + 2,4-D 1 mg/l[12]. At higher concentration of 2, 4-D, the calli turned pale green. Various studies implicated, that the use of TDZ helps in shoot regeneration and proliferation (Hill *et al*., 2006; Kumar *et al*., 2010). Treatment of mature calli with TDZ (0.3 mg/l) for 4 days followed by culturing on MS + BAP 1.5 mg/l + IBA 0.5 mg/l + GA_3 1.5 mg/l resulted in higher shoot regeneration

frequency i.e. 85 %. A pulse treatment of 0.5 mg/l TDZ and 0.1 mg/l IBA for 5 days reported as necessary for shoot organogenesis in green compact callus before sub culturing onto MS medium with 0.5 mg/l BAP and 0.1 mg/l IBA (Misra *et al*., 2010). There were about five distinct shoots per calli. It was reported regeneration of shoot buds (upto seven) on MS + BAP 0.5 mg/l + IBA 0.1 mg/l in about six weeks of incubation, with a regeneration efficiency of 83% (Misra *et al*., 2010). Regenerated shoots were successfully elongated on MS + BAP 0.3 mg/l + GA_3 0.3 mg/l in three weeks time.

In vitro rooting was induced by culturing regenerated shoot lets on MS media containing IAA at 1 mg/l level[6] or NAA at 0.1 mg/l level[4] or IBA at 0.5 mg/l level (Francis *et al*., 2005). In the present study, excised single shoots were rooted in rooting medium (MS supplemented with 0.3 mg/l IBA) after three weeks with a maximum rooting percentage of 35% and the rooted plants were successfully transferred to soil.

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REFERENCES

Das, B. and Velkataiah, B. A. (2000) Minor coumarino-lignoid from *Jatropha gossypifoila.* Biochem. Syst. Ecol. 29, 213-214.

Deore, A.C. & Johnson, T.S. (2008) High-frequency plant regeneration from leaf-disc cultures of *Jatropha curcas* L.: an important biodiesel plant. Plant Biotechnol Rep. 2, 7-11.

Francis, G., Edinger, R. & Becker, K.A. (2005) A concept for simultaneous wasteland reclamation, fuel production, and socioeconomic development in degraded areas in India. Need, potential and perspectives of *Jatropha* plantation. Nat. Resour. Forum. 29, 12-24.

Heller, Physic nut—*Jatropha curcas* L. In: Promoting the Conservation and Use of Underutilized and Neglected Crops 1, International Plant Genetic Resources Institute, Rome, 1996.

Hill, J., Nelson, E., Tilman, D., Polasky, S. and Tiffany, D. (2006) Environmental, economic and energetic costs and benefits of biodiesel and ethanol biofuels. Proc. Natl. Acad. Sci. 103, 11206-11210.

Hussain, A., Virmani, O., Popli, S. P., Misra, L. N., Gupta, M.M., Srivastava, G.N., Abraham, Z. & Singh, A.K. (1992) Dictionary of Indian Medicinal plants, CSIR, New Delhi 263-264.

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

Kaewpoo, M. & Te-chato, S. (2009) Influence of explant types and growth regulators on multiple shoots formation from *Jatropha curcas*, *Science Asia*, 35: 353-357.

Kalimuthu, K.S., Paulsamy, R., Senthilkumar & Sathya, M. (2007) *In vitro* Propagation of the Biodiesel Plant *Jatropha curcas* L., *Plant Tissue Cult. & Bioteh,* 17(2): 137-147.

Kumar, A. & Sharma, S. (2008) An evaluation of multipurpose oil seed crop for industrial uses: A review, Ind. *Crop. Prod*, 28: 1-10.

Kumar, N., Vijay Anand, K.G. & Reddy, M.P. (2010) *In vitro* Plant regeneration of Non-toxic *Jatropha* curcas L.: Direct shoot Organogenesis from Cotyledonary Petiole explants, *J. Crop. Sci. Biotech*, 13(3): 189-194.

Misra, P., Gupta, N., Toppo, D.D., Pandey, V., Misra, M. K. & Tuli, R. (2010) Establishment of long-term proliferating shoots cultures of elite *Jatropha curcas* L. by controlling endophytic bacterial contamination, *Plant Cell Tiss. Org. Cult*, 100: 189-197.

Murashige, T. & Skoog, F.A. (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures, *Plant Physiol*, 15: 473-497.

Openshaw, K. (2000) A review of *Jatropha curcas*; an oil plant of unfulfilled promise, Biomass *Bioenergy*, 19: 1-15.

Rajesh, D., Gupta, A., Mandal, T.K., Singh, D.D., Bajpai, V., Gurav, A. M. & Lavekar, G. S. (2007) Anti-microbial activity of some Medicinal plants, Afr. J. Tradit. Complement. *Altern. Med*, 4: 313-318.

Rajore, S. & Batra, A. (2007) An alternative source for regenerable organic callus induction in *Jatropha curcas* L, *India Journal of Biotechnology*, 6: 545-548.

Shrivastva, S. & Banerjee, M. (2008) *In vitro* clonal propagation of physic nut (*Jatropha curcas* L.): Influence of additives, *Int J. Integr Biol.*, 3: 73-78.

Singh A, Reddy M P, Cjikara J, & Singh S, A simple regeneration protocol from stem explants of *Jatropha curcas*- A biodiesel plant, *Ind. Crop. Prod*, 31 (2) (2010) 209-213.

Sujatha, M. & Mukta, N. (1996) Morphogenesis and plant regeneration from tissue cultures of *Jatropha curcas*, *Plant Cell Tiss. Org. Cult*, 44: 135-141.

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

Yuan, Y., Mei, D., Wang, Z. & Zhang, T. (2008) Combustion and emissions of the diesel engine using bio-diesel fuel, *Front.Mech.Eng.China*, 3:189-19.