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Research Article

IN VITRO SHOOT REGENERATION FROM LEAF DISC CULTURES *JATROPHA CURCAS,* AN IMPORTANT BIOFUEL PLANT

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ABSTRACT
Increased demand of diesel and limited petroleum reserves have intensified research for alternative sources of diesel that is cost effective, less toxic and has less environmental degradation. *Jatropha curcas* (family Euphorbiaceae) is an important biodiesel plant in this regard. In the present study, an efficient micropropagation protocol was established for shoot regeneration from leaf explant of *Jatropha curcas* plant. Adventitive shoots were initiated directly from cultured leaf explants on MS medium supplemented with 1.5 mgl^{-1} BA and 0.1mgl^{-1} IBA. Enhanced shoot multiplication was observed on MS medium with half the NO_3^{2-}concentration supplemented with 0.5 mgl^{-1} BA, 0.1mgl^{-1} IBA and 2.5 mgl^{-1} $AdSO_4$. The effect of liquid and semi-solid media on differentiation of shoot buds was also investigated. Elongated shoots could be rooted on MS medium supplemented with 0.25 mgl^{-1} IBA. Rooted plantlets were successfully acclimatized and transferred to field with 89 % survival percentage.

Keywords: *6-Benzyl Adenine, Jatropha Curcas, Half Nitrate, In Vitro Shoot Regeneration, Liquid Medium*

INTRODUCTION
There is a global effort for developing a viable alternate fuel to mitigate dependence on fossil fuel of petroleum. Extensive research is focused on use of plant oil as a source of biodiesel. As India is an energy deficient country, so there is a lot to gain from cultivation of petro-plants and production of high quality biodiesel for utilization in different sectors (Kumar *et al.*, 2012). *Jatropha curcas* is one such plant that has immense value as a biodiesel crop. Itis one of the most suitable tree borne oilseed for production of biodiesel, both in view of its non-edible oil and prevalence throughout India.

J. curcas (family: Euphorbiaceae) is a soft woody perennial shrub widely spread throughout tropical Asia and Africa as well as from Mexico to Brazil (Openshaw, 2000). In recent years, *Jatropha* has attracted extensive attention of scientists in view of its economic importance, medicinal value and for its seed oil as commercial source of biofuel. Seeds of *Jatropha* contain 30–40% oil with various fatty acids such as palmitic acid, stearic acid, linolenic acid and oleic acid which together constitute 80% of its oil composition. Oil of *Jatropha* has high viscosity and ignition temperature which enables it to be used in lightening and cooking (Misra and Misra, 2010; Kumar and Sharma, 2008).

Jatropha oil has been used as diesel engine fuel, in manufacturing fertilizers, paints, lubricants, soaps, candles and as inhibitor of watermelon mosaic virus (Bisen *et al.*, 2010; Mukherjee *et al.*, 2011). Seed oil is not edible due to toxic and carcinogenic compounds as, curcasine, trypsin inhibitor, phytate and phorbol esters (Verma and Juneja, 2014; Gandhi *et al.*, 1995). Oil contains more oxygen with a higher cetane value with higher combustion quality, clean, non toxic, eco-friendly and economic due to low production cost. It has practical utility as drought resistance suitable for erosion control and is not palatable to grazing animals due to toxicity, thus helpful for eco-restoration in all type of wasteland and also for several medical purposes (Katwal and Soni, 2003; Sharma *et al.*, 2012). In a plantation project carried out in the Institute, it was observed that not all *Jatropha* plants, even those grown in similar conditions of soil and irrigation, were not providing same yield and hence, it was felt necessary to study and identify high oil yielding plants and develop a method to multiply the best saplings identified out of those grown under different agro-conditions through micropropagation.

Research Article

Micropropagation is an important technique of plant tissue culture that has been extensively exploited in the industry for mass propagation of ornamentals, medicinal, timber plants etc. Low shoot multiplication, poor rooting frequency, morphological abnormalities and higher cost of production are among the various factors challenging micropropagation. Most of these problems can be overcome by using specific type and definite concentration of growth regulators while developing efficient micropropagation protocols (Bhojwani and Dantu, 2013).

Cultivation of *J. curcas* assumes utmost importance due to the large scale demand and ensuring large amount of quality material for future use. Conventional propagation of *Jatropha curcas* utilizes seeds and cuttings. But due to poor seed viability, lower germination rate, scanty and delayed rooting of seedling, genetic variability and lower disease resistance hampers practical utility of propagation method (Sujatha and Dhingra, 1993). In order to meet demand of its oil in future, large quantity of planting material is required. Plant tissue culture offers an alternative approach to the plants which are difficult to cultivate, low yield and low cultivation period. Direct or indirect shoot regeneration via callus induction exploits the totipotency of plant cell.

Micropropagation of *Jatropha curcus* has been reported by various authors regarding effect of different concentrations and combinations of plant growth regulators on regeneration from various explants (Sujatha and Mukta, 1996; Khurana-Kaul *et al.,* 2010; Wei *et al.,* 2004; Datta *et al.,* 2007, Deore and Johnson, 2008; Hassan *et al.,* 2014). However, a tissue growth rate and extent and quality of morphogenic response are also influenced by type and concentration of nutrients supplied in culture medium (Niedz and Evens, 2007).

In this study, an *in vitro* micropropagation from leaf discs involving establishment of cultures, multiplication of shoots, rooting and hardening for field transplantation was developed.

MATERIALS AND METHODS

A. Plant Material and Culture Establishment

Jatropha curcus growing in Botanical Garden of the Institute was used as source plant for experiments. Young green, soft nodes (1-2 cm) were excised from mother plant. Explants were soaked in 1% Savlon (Johnson and Johnson, UK) for 8 min and washed under running tap water for 10 min to remove dust particles. This was followed by quick rise with 70% ethanol for 10 s. After evaporating ethanol, explants were surface sterilized with 0.1% mercuric chloride (w/v) solution for 10 min. Explants were subsequently washed 6-7 times with sterile distilled water to remove all traces of mercuric chloride. Nodal (5 mm) and leaf segments (about 1.5 × 1.5 cm^2) were cut and aseptically transferred into MS media supplemented with different auxins (IBA, NAA, 2,4-D and IAA) and cytokinins (BA and Kn). Nodes bearing auxillary buds were cut into segments and were kept upright with base of the nodal segment in contact with medium.

B. Callus Induction

The inoculating leaves were kept in darkness for callus induction and further proliferation. The calli was subcultured with an interval for 4 weeks. The numbers of explants forming calli were scored to calculate calli formation frequency at 8 weeks of culture.

C. Callus Induction and Regeneration of Shoots

Leaf segments (1.5 × 1.5 cm^2) were cut from explant and inoculated on MS media containing 1.5 mgl^{-1} BA with 0.1mgl^{-1} IBA for 4 weeks. Culture medium for induction of adventitious shoot buds consisted of modified MS medium having half-strength nitrate salts (825 mgl^{-1} Ammonium nitrate) supplemented with (i) 1.5 mgl^{-1} BA and 0.1mgl^{-1} IBA, (ii) 0.25 mgl^{-1} BA and 0.1mgl^{-1} IBA and (iii) 0.5 mgl^{-1} BA and 0.1mgl^{-1} IBA.

To study effect of adenine sulphate on shoots proliferation and elongation, callus induced from leaves was inoculated on MS(½NO_3^{2-}) supplemented with 0.5 mgl^{-1} BA, 0.1mgl^{-1} IBA and 2.5 mgl^{-1} $AdSO_4^-$.

D. Media Preparation

For all stages of micropropagation MS (Murashige and Skoog, 1962) basal medium was used either with full strength or with half strength nitrate ((825 mgl^{-1} Ammonium nitrate) supplemented with various

growth regulators. pH of medium was adjusted to 5.7 before adding 0.7% agar (Hi-Media, India). 20 ml medium was poured into 150 × 25 mm rimless borosil culture tubes plugged with polypropylene caps (Polylab) and sterilized at 121˚C and 15 psi for 20 min. The medium was set as slants.

E. Rooting and Acclimatization

Regenerated shoots of about 5-8 cm long were inoculated in MS with 0.25 mg/l IBA for rooting. After 3 weeks of incubation, plantlets were removed from medium, washed in running tap water and transplanted to pro-trays containing sterilized coco peat, which were covered with polythene sheets to maintain high humidity.Transplanted plants were irrigated with MS 1/4th major salt with 2% Bavistin. Survival rates of plants were recorded after 4 weeks. After 4 weeks, the plants were gradually removed from pro-trays and planted in 4" pots in green house conditions.

F. Incubation of Cultures

Callus and shoot cultures were maintained at 25 ± 2 ˚C under a 16 h of photoperiod illuminated with cool fluorescent lamps at an intensity of 30 µmol m^{-2} s^{-1}. Transplanted plants were incubated at 30 °C ± 2°C under a 12 h photoperiod, for 5 weeks.

G. Statistical Analysis

All experiments were in triplicate with n=24. The data were statistically analyzed by using Microsoft Excel (Office 2007, Data Analysis-Descriptive statistics). The analysis of variance and DMRT was done using SPSS version 20.0 software (SPSS Inc, Chicago).

RESULTS AND DISCUSSION

Callusing and Shoot Induction

For multiple shoot induction, leaf explants were inoculated on MS basal medium containing different concentrations of BAP, NAA and IBA. Callus was induced from leaf explants of *Jatropha curcus* on all media used (Table 1) except in the absence of growth regulators. Calli were greenish, non-granular and non-wet on medium containing 0.5mgl^{-1}BA when used alone. In combination with NAA induced greenish, friable and non-wet callus (Figure 1a).

However, MS medium supplemented with 0.5mgl^{-1}Kn and 0.5 mgl^{-1} 2, 4-D yielded highest frequency of callus formation (97 %). Similar results were reported by Attaya *et al.,* (2012) and Rampadarath *et al.,* (2014) using different concentrations of 2, 4-D and BAP. Higher concentration of BA inhibited callus induction. It was reported that multiple shoot formation in *Jatropha* occursin different concentration of cytokinin with auxin (Sahoo and Debata, 1998; Faisal *et al.,* 2003; Sujatha *et al.,* 2005). Of the plant growth regulators used, MS supplemented with 1.5 mgl^{-1} BA and 0.1mgl^{-1} IBA resulted in optimal shoot proliferation.

The combination of BA (0.5 mgl^{-1}) and NAA (0.1mgl^{-1}) induced proliferation of callus but no shoot formation (Table 1). It was reported that inclusions of BA alone or in combination with IAA were not suitable for shoot regeneration in *Jatropha* (Sujatha and Makkar, 2005). The explants cultured on MS basal medium containing 1.5 mgl^{-1} BA and 0.1mgl^{-1} IBA were further used for shoot proliferation studies. The differential response of various explants in shoot regeneration media may be due to different type of plant growth regulator and their concentration used (George, 1993).

Reducing nitrate in MS medium to half significantly increased number of shoots formed compared to full strength nitrate in the same growth regulators of 0.5 mgl^{-1}BAand 0.1mgl^{-1}NAA (Figure 1b). However, no shoot bud formation occurred in MS (½NO_3^{2-}) with 0.5 mgl^{-1} BA and 0.1mgl^{-1} IBA.

The number of shoots per explants was higher when MS (½NO_3^{2-}) with 0.5 mgl^{-1} BA and 0.1mgl^{-1} IBA medium was supplemented with 2.5mgl^{-1} Adenine sulphate (Figure 1c). The average number of shoots buds per explants increased up to 3–4 folds within 8 weeks of initial culture (Figure 1b). In a previous report on shoot proliferation in *Jatropha,* MS medium was supplemented with 22.2 µM BA and 55.6 µM adenine sulphate was used for establishing nodal cultures (Sujatha *et al.,* 2006). Shoot multiplication rate was enhanced in MS medium with 2.3 µMKn, 0.5 µM IBA and 27.8 µM adenine sulphate. Kalimuthu *et al.,* (2007) reported shoot proliferation in nodal explants using MS medium with 1.5 mgl^{-1} BAP, 0.5 mgl^{-1}Kn and 0.1 mgl^{-1} IAA in *Jatropha curcas* species.

Table 1: Response of leaf explants of *Jatropha curcus* to different concentration of plant growth regulators

Medium used	Shoot bud formation	Degree of callusing	Morphogenic Response of callus
MS basal medium	-	-	Callus turns brown and dies
MS + 1.5 mgl^{-1} BA + 0.1mgl^{-1} IBA	++	+++	Callus whitish, granular and compact.
MS + 0.5 mgl^{-1} BA + 0.1mgl^{-1} IBA	+	+	Callus greenish, granular and friable
MS + 0.25 mgl^{-1} BA + 0.1mgl^{-1} NAA	+	++	Callus greenish, nodular, granular and wet
MS + 0.25 mgl^{-1}Kn + 0.5mgl^{-1} IAA	-	+++	Callus creamish, compact, granular and dry
MS + 0.5 mgl^{-1}Kn + 0.5 mgl^{-1} 2,4-D	-	+++	Callus was greenish, granular and friable

+ and - sign indicates degree of callus;(+++ → Profuse callusing; ++ → moderate callusing, + → little callusing; − → no callusing); n= 10

Table 2: Effect of different plant growth regulators on shoot regeneration from leaf explants of *Jatropha curcas*

Initial Medium	Sub culture Medium	shoot bud formation
MS + 1.5 mgl^{-1} BA + 0.1mgl^{-1} IBA	MS + 1.5 mgl^{-1} BA + 0.1mgl^{-1} IBA	1.31^{bc} ± 0.21
MS + 1.5 mgl^{-1} BA + 0.1mgl^{-1} IBA	MS(½NO_3^{2-})+ 0.25 mgl^{-1} BA + 0.1mgl^{-1} IBA	-
MS + 1.5 mgl^{-1} BA + 0.1mgl^{-1} IBA	MS(½NO_3^{2-})+ 0.5 mgl^{-1} BA + 0.1mgl^{-1} IBA	23.43^{a} ± 0.21
MS + 1.5 mgl^{-1} BA+ 0.1mgl^{-1} IBA	MS(½NO_3^{2-})+ 0.5 mgl^{-1} BA + 0.1mgl^{-1} IBA	18.39^{b} ± 2.45
MS + 1.5 mgl^{-1} BAP + 0.1mgl^{-1} IBA	MS + 1.5 mgl^{-1} BA + 0.1mgl^{-1} IBA	12.37^{de} ± 0.61
MS(½NO_3^{2-})+ 0.5 mgl^{-1} BA + 0.1mgl^{-1} IBA + 2.5 mg/l $AdSO_4$	MS(½NO_3^{2-})+ 0.5 mgl^{-1} BA + 0.1mgl^{-1} IBA	13.08^{bcd} ± 0.07
MS(½NO_3^{2-})+ 0.25 mgl^{-1} BA + 0.1mgl^{-1} IBA	MS(½NO_3^{2-})+ 0.5 mgl^{-1} BA + 0.1mgl^{-1} IBA + 0.25 mg/l $AdSO_4$	1.7^{abc} ± 1.42
MS(½NO_3^{2-})+ 0.5 mgl^{-1} BA + 0.1mgl^{-1} IBA	MS(½NO_3^{2-})+ 0.5 mgl^{-1} BA + 0.1mgl^{-1} IBA + 0.25 mg/l $AdSO_4$	39.28^{ab} ± 1.16

Data in the column represent Mean ± SE. Means do not possess same letter are significantly different at 5 % level of probability;

Table 3: Effect of solid and liquid media on in vitro shoot regeneration and elongation in *Jatropha curcas*

Nature of media	No of elongated shoots	Length of shoots (cm)
Liquid medium (without agar)	9.33^{a} ± 0.54	3.42 ± 1.27
Solid medium (with agar)	3.97^{b} ± 0.37	0.68 ± 0.64

Medium used: MS(½NO_3^{2-})+ 0.5 mgl^{-1} BA + 0.1mgl^{-1} IBA + 0.25 mg/l $AdSO_4$; Data in the column represent Mean ± SE. Means do not possess same letter are significantly different at 5 % level of probability; n=

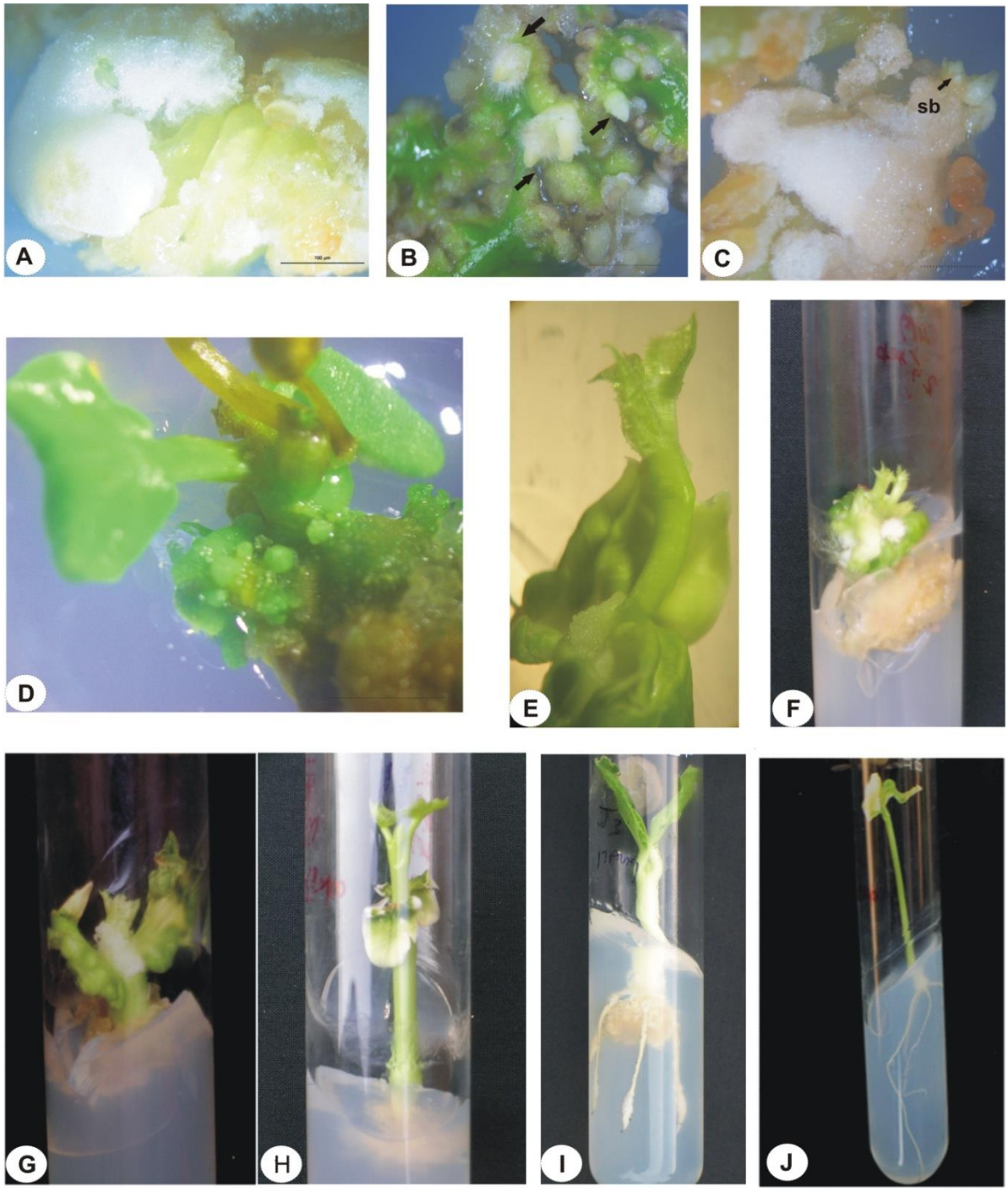

Figure 1: (a). Callus induction in MS with 1.5 mgl^{-1} BAP and 0.1mgl^{-1} IBA (b) Shoot induction in MS (½NO_3^{2-}) with 0.5 mgl^{-1} BA + 0.1mgl^{-1} IBA and 0.25 mg/l $AdSO_4$ (c). Shoot induction in MS (½NO_3^{2-}) with 0.5 mgl^{-1} BA and 0.1mgl^{-1} IBA (d & e). Shoot induction in liquid media (f & h). Shoot induction in semi-solid media (i) Rooting in culture obtained from semi-solid media (j) Rooting in culture obtained from liquid media. Bars = 100µm

A comparative study of effect of solid and liquid media on shoot proliferation was analyzed. Young shoots were cultured in solid or liquid medium containing MS medium (½ ammonium nitrate concentration) supplemented with 0.5 mgl^{-1} BA with 0.1mgl^{-1} IBA and 2.5 mgl^{-1} $AdSO_4$. (Figure 1 d-h).

The liquid medium elicited significantly higher shoot regeneration per explant and shoot length as compared with solid media (Figure 1d), Table 3) at the end of 4 weeks. It was observed in *Piper longum*that liquid medium enhanced shoot elongation and number of shoots as compared to solid media (Rani and Dantu, 2011).

Rooting and Hardening

Among various auxins used, IBA significantly enhanced rooting in *in vitro* regenerated shoots. Use of auxin for rooting in *J.curcas* has been reported by various workers (Shivakumar and Krishnamurthy, 2000; Baskaran and Jayabalan, 2005; Hassan and Khatun, 2010). The regenerated shoots from different media were transferred to root induction medium containing 0.25 mgl^{-1} IBA. Maximum rooting (93 %) was recorded after 3 weeks from shoot differentiated from explants cultured in liquid medium (Figure 1 i-j). The *in vitro* plantlets were transplanted to pro-trays for 4 weeks. The plantlets were further acclimatized and hardened in pots under green house condition. The survival percentage was found to be 89 %, which is much higher as compared to earlier reports (Rajore and Batra 2005).

Conclusion

The results obtained in the present study are quite promising for *in vitro* shoot multiplication from leaf explants *Jatropha curcas*. The shoots obtained from *in vitro* culture developed into young plantlets, which could be successfully acclimatized in field conditions. This protocol may be utilized for large-scale propagation of *Jatropha curcas*, an important biodiesel plant.

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