Libyan Agriculture Research Center Journal International 1 (5): 274-278, 2010
ISSN 2219-4304

Crucial Role of Poor Seed Viability and Superiority of *in vivo* Explant than *In vitro* Derived Explant in Tissue Culture of *Jatropha curcas* L.

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Abstract: An efficient, reliable and reproducible *in vitro* regeneration protocol was standardized by using *in vivo* nodal segments. For shoot initiation, nodal segments inoculated on MS medium augmented with Kn (0.25 mg/l), BAP (0.5 mg/l), IAA (0.25 mg/l) and Adenine sulphate (40 mg/l). After 25 days of incubation, elongation and multiplication was were also observed on the same medium with 9.625±1.372 shoot. After elongation, the shoots were separated and sub-cultured for their *in vitro* root induction on half strength MS medium, supplemented with IBA (2.5 mg/l). The complete plantlets were hardened and acclimatized. Subsequently, these plantlets were transplanted to the natural environment, where they showed 55% survival rate.

Key words: *Jatropha curcas* % Nodal segment % Acclimatized % Incubation and regeneration

Abbreviation: IBA- Indole-3-butyric acid % MS medium- Medium Murashige and Skoogs medium $AdSO_4$ – - Adenine sulphate % IAA- Indole % -3- acetic acid % BAP- 6- Benzylamino purine and Kn - 6- furfurylamino purine

INTRODUCTION

Jatropha curcas L., commonly known as Physic nut, Ratanjyot is a stress resistant, perennial and monoecious plant belonging to the family Euphorbiaceae.

The plant has immense environmental, medicinal and economic values. It is an anti-feedant, fast growing and is used as a hedge plant by farmers all over the world [1]. It is also an excellent species for agroforestry. has great potential in curing various diseases like rheumatism, scabies, eczema, paralysis, malaria, leprosy, piles, snakebite, cancer, tumor, HIV etc. [2-8]. The drug obtained from *J. curcas* is Dravanti [9], which has anthelmintic properties. *J. curcas* also have the anti-cancerous properties because of the presence of alkaloid Jatrophine in latex [6].

Keeping in view the immense economic and medicinal, value, social, as non food crop, perennial nature, faster rate of growth, suitability to various climatic and soil conditions, this plant is most preferred over other biofuel crops like soybean, sunflower, corn, maize etc. At the same time, tissue culture is needed of this plant for saving the time, space and labour intensive etc. Traditional propagation system need a bulk of seeds and stem cuttings for its regeneration, but propagation through seeds is dependent on rainfall, high moisture, sowing time and depth of sowing the seeds etc. Further, the seeds of this plant shows poor seed viability, low germination, scanty and delayed rooting of seedlings and stem cuttings even after having the above said conditions for seed propagation [10-12].

A number of regeneration protocols regarding the tissue culture of *Jatropha curcas* have been developed by using different *in vitro* explants like shoot-tip, epicotyls, hypocotyls, petiole and leaf explants [13-15]. All these protocols were dependent on *in vitro* seedling, which can only be provided by seed germination in *in vitro* conditions. Keeping its seed viability problem, the present research endeavour was undertaken we have considered this problem of seed germination and decided to micropropagate this particular plant species using *in vivo* nodal segment, so as to fulfill the continuous demand of pharma and oil producing companies.

MATERIALS AND METHODS

Plant Source: Nodal segments of *Jatropha curcas* (Voucher specimen number-RUBL20831 and the identification of this plant was done by Prof. S. Mishra (Retd.), Taxonomist, Department of Botany, University of Rajasthan, Jaipur) were collected from 3-5 years old tree growing in the botanical garden of Rajasthan University,

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Jaipur. Nodal segments taken from *in vivo* grown plant were thoroughly washed in running tap water for 20 minutes then rinsed with teepol 0.2% (v/v) for 3-5 minutes followed by proper washing with sterilized double distilled water for 3-4 times to remove all the traces of sterilant. Before inoculation of these explants, they were again sterilized with 0.1% mercuric chloride ($HgCl_2$, w/v) for 30 seconds and then rinsed with double distilled water at least thrice. These double sterilized nodal segments were cultured on various concentrations of growth hormones supplemented in MS medium.

Culture Media and Conditions: The sterilized explants inoculated on Murashige and Skoog media [16] containing 3% sucrose and 0.8% agar along with various concentrations of different growth regulators like Kn, BAP, IAA, IBA, NAA, 2,4-D etc. The pH of the medium was adjusted to 5.8±0.02 prior to autoclaving at 15 psi, 121°C for 20 minutes. After the preparation and solidification of media, the sterilized explants were inoculated on the medium and the cultures were incubated at 25±2°C, 55±5% humidity and 16/8 hours photo and dark period, respectively. For each experiment, 8 replicates were prepared and each protocol was repeated at least thrice.

Shoot Proliferation and Multiplication: For *in vitro* shoot multiplication from nodal explants, various concentrations of cytokinins i.e. Kn / BAP (0.10- - 6.0 mg/l) alone as well as in combination were tested. The optimized Kn and BAP were again tested with various concentrations of different auxins like IAA, IBA, NAA and 2, 4-D (0.1 – 2.0 mg/l), respectively. Effects of various additives (Adenine Sulphate, Ascorbic acid, Citric Acid, Charcoal and Proline) were also taken into account for the experiment. The combination of Kn (0.25 mg/l), BAP (0.5 mg/l), IAA (0.25 mg/l) and Adenine Sulphate (40 mg/l) produced 9 to 10 shoot per node within 3--4 weeks. The initiated shoots were then subsequently subcultured on the same medium for their elongation and further multiplication.

Rooting and Hardening: The *in vitro* elongated shoots were then aseptically transferred onto half strength MS medium supplemented with various concentrations of (1.0- - 6.0 mg/l) different auxins (IAA, IBA, 2,4-D, NAA) alone. IBA at a concentration of 2.5 mg/l proved to be the best for rooting. The plantlets were then gently picked up from the culture vessels and rinsed with distilled water and transferred to polycups containing vermicompost and autoclaved soil (1:3). Polycups were covered with inverted glass beakers to maintain high humidity and kept in culture chamber for their hardening. They were then gradually exposed to natural conditions for their acclimatization.

Statistical Analysis: A completely randomized design was used in all the experiments. For statistical analysis, mean±S.E were calculated. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level.

RESULTS AND DISCUSSION

During the present research work, the effect of phytohormones including cytokinins (Kn and BAP alone or in combination), auxin (IAA) and an additive (Adenine sulphate) has been quantified in response to get multiple shoots in *Jatropha curcas* L (Fig A). Cytokinin (Kn and BAP) separately incorporated in the MS medium gave meanger number of shoots (4.025±0.270 and 4.00±0.327).

In consonance to the results obtained on Kn, Paranhos *et al*. [17] also reported multiple shoots on Kn supplemented MS medium in *Psychotria umbellate* Vell. In contrast to the results obtain, Sen *et al*. [18] established multiplication of shoots on MS medium supplemented with BAP in *Phyllanthus amarus* Schum. and Thonn. In addition to results obtained on Kn, BAP played an important role to stimulate the proliferation of axillary buds, their further growth and finally multiple shoot proliferation in *J. curcas*. At the same time, Kalidass and Mohan [19] also obtained multiple shoots on BAP incorporated MS medium in *Phyllanthus urinaria* Linn, also favored the results obtained by the author. In contrast to this, Chakraborthy *et al*. [20] showed the multiplication of shoots through axillary buds in *Podophyllum hexandrum* on MS medium along with IAA.

During the present course of experimentation neither Kn nor BAP alone gave optimum multiplication of shoots. Here, a combination of Kn and BAP (0.25 mg/l and 0.50 mg/l) found to be optimum for getting maximum number of shoots up to 5.625±0.245 within 3 to 4 weeks of inoculation (Table 1). A number of scientists favored the results on multiplication of shoots on a combination of BAP and Kn in *Withania somnifera* L. Dunal, *Phyllanthus niruri* L. and *Vitex agnus-castus* [21-23], respectively. In contrary to the results on combination of Kn and BAP, another combination of different cytokinins along with Adenine sulphate (BAP+TDZ+$AdSO_4$) has been reported best to stimulate shoot production in *Acacia sinuata* (Lour.) Merr. [24]. Further, BAP and TDZ in combination also reported optimum for multiplication of shoots in *Psoralea corylifolia* [25].

Table 1: Effect of cytokinins on shoot bud proliferation through axillary meristems in Jatropha curcas L

Cytokinins level (mg/l)		
Kn	BAP	Number of shoot buds per explants*Mean±S.E
0	0	Nil
0.10		3.775±0.260
0.25		4.025±0.270
0.50		3.150±1.610
1.0		3.625±0.420
3.0		1.50±0.189
6.0		Nil
	0.10	3.250±0.250
	0.25	3.750±0.313
	0.50	4.00±0.327
	1.0	3.750±0.252
	3.0	1.750±0.250
	6.0	1.075±0.183
0.25	0.25	5.110±1.11
0.25	0.50	5.625±0.245
0.25	1.0	4.125±0.398
0.25	1.50	5.500±0.267
0.25	2.0	2.250±0.025
0.25	3.0	Nil

*Values represent mean±SE of 28 replicates/treatment in three repeated experiments. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level

Table 2: Effect of cytokinins (Kn (0.25mg/l) + BAP (0.5mg/l)) with various auxins on axillary bud proliferation through axillary meristem in Jatropha curcas L

IAA	IBA	NAA	2,4-D	Number of shoot buds per explant*Mean±S.E
0.10	-	-		4.750±0.313
0.25	-	-		6.625±0.295
1.00	-	-		4.250±0.180
2.00	-	-		1.100±0.250
	0.10	-		1.778±0.278
	0.25	-		1.375±0.183
	1.00	-		1.750±0.267
	2.00	-		Nil
		0.10		2.250±0.313
		0.25		1.500±0.189
		1.00		1.875±0.295
		2.00		Nil
			0.10	1.250±0.164
			0.25	1.875±0.295
			1.00	1.375±0.183
			2.00	1.225±0.183

*Values represent mean±SE of 28 replicates/treatment in three repeated experiments. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level

Table 3: Effect of Adenine sulphate on axillary bud proliferation through axillary meristems in Jatropha curcas L

Adenine Sulphate(mg/l)	Number of shoot buds per explant *Mean±S.E
10	5.750±0.295
20	6.500±0.164
30	7.875±0.324
40	9.625±1.372
50	6.125±0.295
60	5.125±0.164

*Values represent mean±SE of 28 replicates/treatment in three repeated experiments. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level

Auxin especially IAA in the present study at 0.25 mg/l enhanced the rate of multiplication beyond 5.625±0.245 to 6.625±0.295 (Table 2) as mentioned previously in the case of *Memecylon edule*, *Prosopis cineraria* (L.) Druce and *Melia azedarach* L. [26-28]. On the other hand, NAA in combination with BAP stimulated increment in the number of shoots in case of *Emblica officinalis*, *Neoglaziovia variegate* and *Acacia nilotica* L. [29, 30], respectively.

Adenine sulphate in plant tissue culture plays an important role in division of cells, their elongation, to stimulate production of chlorophylls in cells. The basic nature of $AdSO_4$ was encashed in the present work to maximize the number of shoots from 6.625±0.295 to 9.625±1.372 (Table 3) within 3 to 4 weeks of fresh inoculation, when the fresh nodal segments were placed on MS medium supplemented with Kn (0.25 mg/l), BAP (0.50 mg/l), IAA (0.25 mg/l) and $AdSO_4$ in combination. $AdSO_4$ was proved to be best for shoot enhancement in *Thevetia peruviana* (Pers.) Schum. [31], *Cichorium intybus* L.cv. Focus [32] and *Ophiorrhia prostrate* D. Don [33]. In contrast to $AdSO_4$, L-asparagine, L-proline, L-glutamine and serine also proved beneficial for shoot multiplication in *Sorghum bicolor* (L.) Moench. [34]. On this medium and plant growth hormones multiple shoots were elongated meanwhile the shoot multiplication phase (Fig B and C).

Table 4: Root induction in in vitro regenerated shoots of Jatropha curcas L

IAA	IBA	NAA	2,4-D	%Rooting response	Other responses
1.0-6.0				40-50%	Swollen and small roots
	0.10 1.0			Nil	Profuse, long roots with no callus at the base
	2.0			30-40%	
	2.5			70-80%	
	3.0-6.0			40-50%	
		1.0-3.0		Nil	Profuse callus at the base
		4.0-6.0		30-40%	Slight callusing at the base with small roots
			1.0-6.0	Nil	Shoots turned brown with no root development

*Values represent mean±SE of 28 replicates/treatment in three repeated experiments. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level

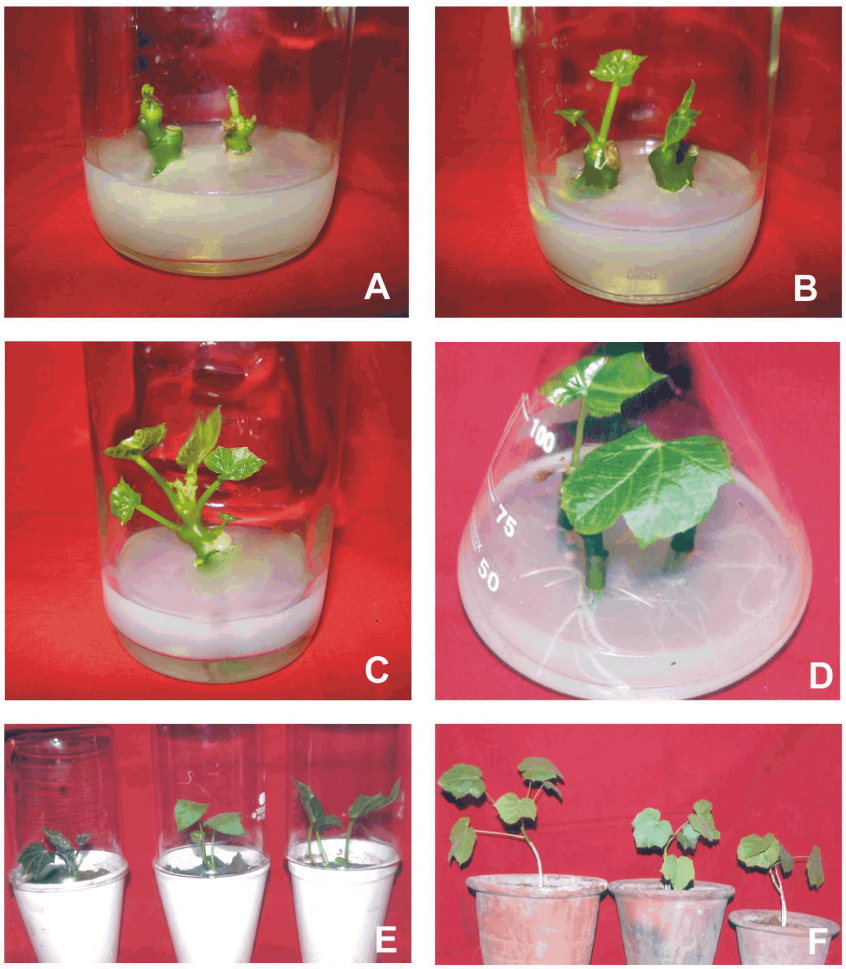

Fig. A- Shoot initiation from nodal explant on MS medium supplemented with Kn (0.25 mg/l), BAP (0.5 mg/l) IAA(0.25 mg/l) and $AdSO_4$; Fig. B- Shoot formation on same medium in 2 weeks; Fig. C- Multiple shoot formation in 3-4 weeks on same medium; Fig. D- Root formation on ½ MS medium supplemented with IBA (2.5 mg/l); Fig. E- Complete plantlet transfer in polycups covered with beaker; Fig F- Acclimatized plantlets.

For *in vitro* rooting, 3-4 cm long shoots were transferred to ½ strength MS medium in combination with IBA (2.5 mg/l). Profuse, reticulate roots showing 70-80% response were obtained on the above concentration (Table 4 and Fig D). Similar results were reported in *Withania somnifera* L. Dunal [35]; and in *Acacia senagal* [36]. In contrast to this IAA induced rooting in several plants as reported in *Azadirachta indica* A Juss. [37] and in *Hypericum maculatum* Cranz [38].

These *in vitro* developed plantlets were allowed to harden and acclimatize to the natural soil and environmental conditions (as mentioned in 'Material and Methods') (Fig. E and F). These tissue cultured plantlets after transplantation to the field show 55% survival rate. During the present studies around 55% survival rate was observed (Fig E and F).

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