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EVALUATION OF *IN VITRO* RESPONSES FROM DIFFERENT EXPLANTS OF ELITE *JATROPHA CURCAS* L.

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SUMMARY

***Jatropha* is a genus of approximately 175 succulents, shrubs and trees (some are deciduous like *Jatropha curcas* L) from the family Euphorbiaceae. Seeds, besides being a source of oil for biodiesel, can also be used for manufacturing other useful products such as candles, high quality soaps and cosmetics, and other herbal products. Since *J. curcas* is primarily a cross pollinated crop, vegetative propagation is important to maintain genetic purity of the elite lines and transformants. A regeneration protocol was optimized for the faster propagation of elite jatropha plant. Out of different explants tested (petiole, apical bud and leaf), apical buds were found to be the best for callus induction. Shoot regeneration from calli induced from petioles was the best. Various combinations of auxins with cytokinins were suitable for callus induction. The best shoot regeneration (75 %) was in MS medium supplemented with IBA (1.23µM) and BAP (6.6µM). Root induction (100%) was successfully obtained in MS and ½ MS medium. Acclimatization and hardening was quite successful with survival rate of 75 per cent.**

***Key words*: Apical bud, BAP, IBA, *Jatropha curcas*, petiole**

INTRODUCTION

Jatropha curcas is a small tree or large shrub with smooth gray bark, which exudates a whitish colored watery latex upon cut. It belongs to the family Euphorbiaceae. *Jatropha curcas* has unique pride among the various plants because of its multiple uses like ethanomedical value, ornamental value, carbon sequestration, comparatively good fuel properties etc. Almost all parts of the plant have important pharmacological effect and are used in traditional medicine (Staubmann *et al.* 1999). Sujata and Mukta (1996) developed tissue culture protocols for the propagation of *Jatropha curcas*. However, Sardana *et al*. (1998) reported that propagation of this plant species through tissue culture is relatively difficult, due to latex containing shrub, which makes it recalcitrant for tissue culture (Sardana *et al.* 1998).

Due to high oil content and toxic nature, Jatropha oil has been recommended for biodiesel. A lot of emphasis world over has been placed on energy plantation to overcome carbon related issues along with a potential of source of bioenergy. As such, genetically improved and naturally evolved plants are being collected from various geographical locations. Molecular analysis for different important genes and many drawbacks of the plants for different environments are noticed for plantation

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strategies. In view of this, and to promote plantation economics, selections have been made predominantly based on plant performance at Crop Research Center, Pantnagar.

MATERIALS AND METHODS

Different explants (apical buds, petioles and leaves) were collected for standardization of regeneration protocol of *Jatropha curcas* L. from the 5 years old Jatropha plants at Crop Research Centre, Pantnagar. Collected explants were sterilized with Tween-20, alcohol, 2 % sodium hypochlorite (1-1.2 % available chlorine). It was then inoculated in the appropriate medium. Cutting edges of the explants should make a direct contact with medium. The basal MS medium (Murashige and Skoog 1962) was used with derived supplementation of phytoregulators for callusing, and induction of shoots and roots . The medium was supplemented with different auxins: IBA (1.23-14.76μM), NAA (1.34-16.08μM), IAA (0.72-1.72μM) and 2,4-D (1.13-13.56μM) and cytokinins: BAP (0.44-19.6μM), adenine (0.53-21.17μM) and kinetin (0.57-18.60μM). The rooted shoots were removed from jam bottles and washed with sterile water to remove traces of agar sticking on the roots and dipped in 0.2 % (W/V) bavistin solution for 20 min. Shoots were transferred to plastic pots filled with sterile soil and kept in glass house. Initially, the pots were covered with polybags. After 15 days, they were uncovered and shifted gradually from shade to sunlight.

Experiments were set up in Completely Randomized Design with 9 replicates per treatment. Observations for the number of explants forming callus, shoots and number of roots per responding explants/callus for direct and indirect regeneration were recorded by visual observations. The experimental design for testing the significance of mean and interaction effects of media (callusing/shooting/rooting) and different explants (for callusing and morphogenesis) were Three Factorial Completely Randomized Design.

RESULTS AND DISCUSSION

Morphogenic calli were induced successfully at the selected concentrations of IBA and BAP, varying between 4.92μM to 14.76μM for IBA and 0.44μM to 2.20μM for BAP, respectively. It was observed that the percentage of callus induction varied from 40 to 85%. IBA (4.92μM) with BAP (1.33μM), IBA (4.92μM) with BAP (1.76μM) and IBA (7.38μM) with BAP (0.44μM) lead to 85% callus induction frequency in case of petiole, which decreased to 40% in media containing IBA (7.38μM) with BAP (1.76μM) in case of leaves (Table 1). Calli were initiated in MS medium supplemented with NAA (5.36-16.08μM) in combination with BAP (0.88-2.20μM). The inductive frequency of callusing ranged from 50 to 85%. Maximum callus induction was observed in the presence of NAA (5.36μM) + BAP (1.33/1.76/2.20μM) and NAA (8.04μM) + BAP (0.44/0.88/1.33/1.76μM), whereas, minimum response was observed in case of NAA (5.36μM) + BAP (2.20μM) (Table 2). In all the above combinations tried, callus induction from petiole, apical bud and leaf explants started developing first on both cut ends after 6 days of inoculation (for apical buds), 8 days for petioles and 10 days for leaf. Callus were successfully induced on MS medium supplemented with IBA + adenine, NAA + adenine or IAA + BAP (data not shown). Apical buds and petioles produced white compact calli, whereas, leaf produced green compact callus. Callus induction frequencies were highest in case of apical buds and minimum in case of leaves. But no callus was induced on growth regulator-free MS medium (data not shown).

Calli were inoculated in media containing various concentrations of kinetin, adenine and BAP with NAA, IBA and IAA. Of the three cytokinins tested, BAP was most effective in multiple shoot formation. Caulogenic ability of explants was exhibited at a wider range of growth regulator combinations for BAP (2.20-17.6μM) + IBA(1.23-2.46μM) and NAA (1.34-2.68μM). Shoot induction frequencies varied from 30 to 75% (Table 2). Maximum shoot induction frequency was 75%, which decreased to 30% for petiole. Maximum number of shoots per callus(4) was recorded in IBA (1.23μM) + BAP (6.60μM), whereas none in the case of IBA (1.23μM) + BAP (2.20/11.0/13.2/15.4μM), NAA (1.34μM) + BAP (11.0μM) and NAA (2.68μM) + BAP (0.88/8.8μM). *De novo* shoot formation through callus derived from the petiole and apical buds was initiated within 12-15 days, and all the shoots were healthy and

Table 1. Influence of different concentrations of IBA/NAA and BAP on callusing from different explants of *J. curcas.*

Combination (µM)	Days for callus initiation			Callus induction frequency (%)			Texture of Callus		
	P	AB	L	P	AB	L	P	AB	L
$I_{4.92}B_{0.88}$	-	6-8	-	-	80	-	-	White compact	-
$I_{4.92}B_{1.33}$	8-10	6-8	-	70	85	-	White compact	White compact	-
$I_{4.92}B_{1.76}$	8-10	6-8	10-15	70	85	60	White compact	White compact	Green compact
$I_{4.92}B_{2.20}$	8-10	6-8	10-15	65	80	50	White compact	White compact	Green compact
$I_{7.38}B_{0.44}$	8-10	8-10	10-15	70	85	50	White compact	White compact	Green compact
$I_{7.38}B_{0.88}$	9-12	8-10	12-16	70	80	50	White compact	White compact	Green compact
$I_{7.38}B_{1.33}$	9-12	8-10	12-16	65	80	50	White compact	White compact	Green compact
$I_{7.38}B_{1.76}$	9-12	8-10	12-16	60	80	40	White compact	White compact	-
$I_{7.38}B_{2.20}$	9-12	10-15	-	60	75	-	White compact	White compact	-
$I_{9.84}B_{0.44}$	9-12	10-15	-	60	80	-	White compact	White compact	-
$I_{9.84}B_{0.88}$	10-15	10-15	-	60	80	-	White compact	White compact	-
$I_{9.84}B_{1.33}$	10-15	10-15	-	70	70	-	White compact	White compact	-
$I_{9.84}B_{1.76}$	10-15	10-15	-	65	70	-	White compact	White compact	-
$I_{9.84}B_{2.20}$	10-15	-	-	60	-	-	White compact	-	-
$I_{12.30}B_{0.44}$	10-15	-	-	50	-	-	White compact	-	-
$I_{12.30}B_{0.88}$	10-15	10-15	-	50	60	-	White compact	White compact	-
$I_{12.30}B_{1.33}$	14-16	-	-	50	-	-	White compact	-	-
$I_{12.30}B_{1.76}$	14-16	-	-	50	-	-	White compact	-	-
$N_{5.36}B_{1.33}$	9-12	7-9	-	75	85	-	White compact	White compact	-
$N_{5.36}B_{1.76}$	9-12	7-9	10-12	80	85	60	White compact	White compact	Green compact
$N_{5.36}B_{2.20}$	9-12	7-9	10-12	80	85	50	White compact	White compact	Green compact
$N_{8.04}B_{0.44}$	9-12	7-9	-	85	80	-	White compact	White compact	-
$N_{8.04}B_{0.88}$	9-12	7-9	-	85	80	-	White compact	White compact	-
$N_{8.04}B_{1.33}$	10-13	8-10	-	85	85	-	White compact	White compact	-
$N_{8.04}B_{1.76}$	10-13	8-10	-	80	85	-	White compact	White compact	-
$N_{8.04}B_{2.20}$	10-13	8-10	-	75	65	-	White compact	White compact	-
$N_{10.72}B_{0.44}$	10-13	8-10	-	65	65	-	White compact	White compact	-
$N_{10.72}B_{0.88}$	10-13	8-10	-	70	65	-	White compact	White compact	-
$N_{10.72}B_{1.33}$	10-13	8-10	-	65	55	-	White compact	White compact	-
$N_{10.72}B_{1.76}$	10-13	-	-	60	-	-	White compact	-	-
$N_{10.72}B_{2.20}$	10-15	-	-	55	-	-	White compact	-	-
$N_{12.30}B_{0.44}$	10-15	-	-	55	-	-	White compact	-	-

Source	SE of mean	CD (1% level of significance)	Source	SE of mean	CD (1% level of significance)
Explants	0.3857460	1.4230	Explants	0.5336666	1.9687
NAA	0.4979960	1.8371	IBA	0.6889606	2.5416
BAP	0.4979960	1.8371	BAP	0.6889606	2.5416
Explants* NAA	0.8625543	3.1820	Explants* IBA	1.1933147	4.4023
NAA*BAP	0.8625543	3.1820	IBA *BAP	1.1933147	4.4023

I-IBA, B-BAP, N-NAA, L-Leaf, AB-Apical bud, P-Petiole. Each numerical value is the mean of three replicates (each replication consisted of three jam bottles), Ineffective treatments are not included, All numerical values are in integers, Numerical values in subscript are in µM, The mean effects of callus induction frequencies for different explants and interaction between explants and phytohormones were found to be highly significant at 1 % level of significance

Table 2. Effect of IBA/NAA and BAP on shoot formation from callus derived from petiole of *J. curcas*

Combination	Average no. of shoots/callus		Regeneration frequency (%)	
	P	AB	P	AB
$I_{1.23}B_{2.20}$	2	-	40	-
$I_{1.23}B_{4.40}$	3	2	50	60
$I_{1.23}B_{6.60}$	4	2	70	75
$I_{1.23}B_{8.80}$	3	1	65	70
$I_{1.23}B_{11.0}$	2	-	50	-
$I_{1.23}B_{13.2}$	2	-	40	-
$I_{1.23}B_{15.4}$	1	-	30	-
$I_{2.46}B_{4.40}$	1	1	50	40
$I_{2.46}B_{6.60}$	3	1	60	60
$N_{1.34}B_{4.40}$	3	1	70	60
$N_{1.34}B_{6.60}$	3	2	75	60
$N_{1.34}B_{8.80}$	2	1	60	50
$N_{1.34}B_{11.0}$	2	-	50	-
$N_{2.68}B_{2.20}$	1	-	50	-
$N_{2.68}B_{4.40}$	1	1	50	60
$N_{2.68}B_{6.60}$	2	2	60	70
$N_{2.68}B_{8.80}$	1	-	50	-

I-IBA, B-BAP, N-NAA, P-Petiole, AB-Apical bud, Each numerical value is the mean of three replicates (each replication consisted of three jam bottles), Ineffective treatments are not included, All numerical values are in integers, Numerical values in subscript are in µM, The mean effects of shoot induction frequencies for different explants and interaction effects between explants and phytohormones were found highly significant at 1 % level of significance

attained a average height of 2.5-3.5 cm after 20 days. Shoots from apical buds were surrounded by white callus and those from petioles were slight green. Shoots were separated and subcultured on the same media for further multiplication.

Combinations of NAA with adenine, IBA with adenine, IAA with BAP and IAA with kinetin failed to induce shoot formation (data not shown). After 3 weeks, elongated shoots were separated individually and transferred to rooting medium on growth regulator-free, full strength MS medium, half strength MS medium and medium supplemented with IBA and 2,4-D. Elongated normal roots were formed within 15-20 days. Rooting frequency varied from 80 % (IBA:2.46/4.92µM, 2,4-D: 2.26/4.52µM) to 100 % in MS and ½ MS media (Table 3).

Table 3. Effect of varying medium constituents on regenerated shoots of *J. curcas* for root formation

Combination	Days for root initiation	Rooting frequency (%)
MS	15-17	100
½ MS	15-17	100
$I_{2.46}$	18-20	80
$I_{4.92}$	18-20	80
$D_{2.26}$	18-20	80
$D_{4.52}$	18-20	80

In a subsequent direct regeneration experiment, effect of different concentrations of NAA and IBA with BAP was compared to optimize the best growth regulator combination for callusing and shoot and root regeneration form petiole, apical bud and leaf as explants. Table 4 showed successful morphogenesis response from different explants of *J. curcas*. Callus induction frequencies varied from 50 to 90%. Apical buds and petiole showed higher callus induction frequencies as compare to leaf explants. The caulogenic inductive frequency ranged from 50 to 85 %. Shoot induction was maximum in the presence of IBA (2.46µM) + BAP (8.8µM) and minimum in presence of IBA (4.92µM) + BAP (2.20µM). Root induction in petiole and apical buds varied from 60 to 80%. Profuse rooting was obtained in all combinations. Higher auxin to cytokinin ratio leads to adverse effect on root induction frequency. In all the above combinations, callusing was maximally induced in case of apical buds, followed by petioles, while it was minimum in case of leaves. Callus morphogenesis could not occur in case of leaf explants. Regenerated shoots were healthy and rooting was profuse.

The regeneration study revealed the capacity of *Jatropha curcas* to regenerate plantlets from petioles, apical buds and leaf explants. Regeneration occurred

Table 4. Morphogenic response of various explants of *J. Curcas* on MS medium supplemented with different concentrations of IBA/NAA and BAP

Combination	Days for callus induction			Days for shooting			Days for rooting			Callus induction frequency (%)			Root induction frequency (%)			Shoot induction frequency (%)		
	P	AB	L	P	AB	L	P	AB	L	P	AB	L	P	AB	L	P	AB	L
$I_{2.46}B_{2.20}$	8-10	-	-	12-15	-	-	20-22	-	-	80	-	-	80	-	-	60	-	-
$I_{2.46}B_{4.40}$	8-10	8-10	-	12-15	15-18	-	20-22	18-22	-	75	75	-	80	80	-	80	70	-
$I_{2.46}B_{6.60}$	8-10	6-8	-	15-18	15-18	-	20-22	18-22	-	70	70	-	80	70	-	80	75	-
$I_{2.46}B_{8.80}$	6-8	6-8	6-8	15-18	15-18	-	20-22	18-22	-	60	70	60	75	60	-	85	75	-
$I_{2.46}B_{11.0}$	6-8	6-8	6-8	15-18	15-18	-	22-24	20-22	-	60	70	60	70	60	-	70	70	-
$I_{2.46}B_{13.2}$	6-8	8-10	8-10	18-20	18-20	-	22-24	20-22	-	60	70	60	70	60	-	60	60	-
$I_{4.92}B_{2.20}$	8-10	8-10	-	15-18	18-20	-	20-22	18-20	-	90	90	-	85	80	-	50	50	-
$I_{4.92}B_{4.40}$	8-10	6-8	-	18-20	15-18	-	20-22	18-20	-	90	90	-	80	75	-	75	70	-
$I_{4.92}B_{6.60}$	6-8	6-8	6-8	18-20	15-18	-	22-24	18-20	-	85	80	70	80	70	-	80	70	-
$I_{4.92}B_{8.80}$	6-8	6-8	8-10	18-20	18-20	-	22-24	16-18	-	80	75	70	80	70	-	70	75	-
$I_{4.92}B_{11.0}$	8-10	6-8	-	20-22	18-20	-	22-24	18-20	-	80	70	-	80	70	-	60	75	-
$I_{4.92}B_{13.2}$	-	-	-	20-22	-	-	22-24	-	-	-	-	-	-	-	-	60	-	-
$N_{2.68}B_{2.20}$	9-11	-	-	14-16	-	-	-	-	-	80	-	-	75	-	-	60	-	-
$N_{2.68}B_{4.40}$	9-11	9-11	12-15	14-16	16-18	-	22-24	20-22	-	75	75	50	75	75	-	80	70	-
$N_{2.68}B_{6.60}$	9-11	7-9	12-15	16-18	16-18	-	22-24	20-22	-	70	65	50	75	65	-	85	70	-
$N_{2.68}B_{8.80}$	7-9	7-9	12-15	16-18	16-18	-	22-24	20-22	-	70	70	65	70	55	-	85	75	-
$N_{2.68}B_{11.0}$	7-9	7-9	12-15	16-18	16-18	-	24-26	22-24	-	65	70	65	65	55	-	70	70	-
$N_{2.68}B_{13.2}$	7-9	9-11	-	20-22	20-22	-	24-26	22-24	-	90	65	-	65	55	-	65	60	-
$N_{2.68}B_{2.20}$	9-11	7-9	-	16-18	20-22	-	22-24	20-22	-	90	85	-	80	75	-	50	50	-
$N_{2.68}B_{4.40}$	9-11	9-11	14-16	20-22	16-18	-	22-24	20-22	-	80	85	50	75	70	-	70	75	-
$N_{2.68}B_{6.60}$	7-9	7-9	14-16	20-22	16-18	-	24-26	20-22	-	80	85	50	75	65	-	80	70	-
$N_{2.68}B_{8.80}$	7-9	9-11	-	20-22	20-22	-	24-26	18-20	-	75	80	-	70	65	-	65	75	-
$N_{2.68}B_{11.0}$	9-11	9-11	-	22-24	20-22	-	24-26	18-20	-	70	80	-	70	60	-	65	75	-
$N_{2.68}B_{13.2}$	-	-	-	22-24	-	-	-	20-22	-	-	-	-	-	-	-	-	-	-

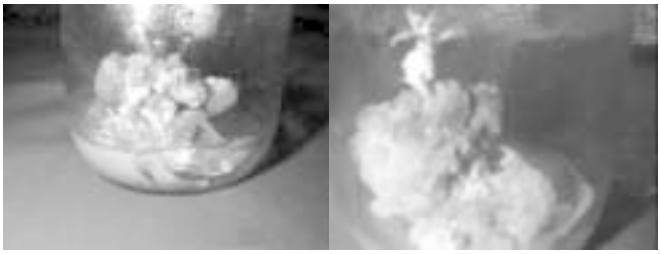
Source	Callus induction (IBA and BAP)		Shoot induction(IBA and BAP)		Root induction (IBA and BAP)	
	SE of mean	CD at 1% level of significance	SE of mean	CD at 1% level of significance	SE of mean	CD at 1% level of significance
Explants	1.3327	4.9856	0.9084	3.3982	1.0598	3.9649
IBA	1.0881	4.0707	0.7417	2.7747	0.8653	3.2373
BAP	1.8847	7.0508	1.2846	4.8059	1.4988	5.6072
Explants*IBA	1.8847	7.0508	1.2846	4.8059	1.4988	5.6072
IBA*BAP	3.2643	12.2123	2.2250	8.3240	2.5960	9.7120
Explants*BAP	2.6653	9.9713	1.8167	6.7965	2.1196	7.9298
Explants*IBA*BAP	4.6165	17.2709	3.1466	11.7720	3.6713	13.7349
Explants	1.3327	4.9856	0.9084	3.3982	1.0598	3.9649
IBA	1.0881	4.0707	0.7417	2.7747	0.8653	3.2373
BAP	1.8847	7.0508	1.2846	4.8059	1.4988	5.6072
Explants*IBA	1.8847	7.0508	1.2846	4.8059	1.4988	5.6072
IBA*BAP	3.2643	12.2123	2.2250	8.3240	2.5960	9.7120
Explants*BAP	2.6653	9.9713	1.8167	6.7965	2.1196	7.9298
Explants*IBA*BAP	4.6165	17.2709	3.1466	11.7720	3.6713	13.7349

I-IBA, B-BAP, N-NAA, AB-Apical bud, P-Petiole, L-Leaf, Each numerical value is the mean of three replicates (each replication consisted of three jam bottles), Ineffective treatments are not included, All numerical values are in integers, Numerical values in subscript are in μM, The mean effect of callus/shoot/root induction frequencies for different explants and interaction effects between explants/ callus and shoots were found to be highly significant at 1 per cent level of significance.

directly and indirectly as well. During direct regeneration, morphogenesis was induced on the surface of petiole and apical buds and during indirect regeneration, regenerated shoots were differentiated from calli followed by rooting (Fig. 1, A-E).

Callus induction from petiole, apical bud and leaf explants was readily obtained in different phytohormone combinations. Growth regulators are important factors, which can selectively influence the genes to trigger differentiation of cells in culture (Thorpe and Stefania 1981, Thorpe 1983). Apical buds showed maximum callus induction frequency (85 %) followed by petioles. The suitability of apical bud explants for regeneration and its sensitivity to various hormones may be due to actively dividing meristematic cells (Rajore and Batra 2005). The variations in plant regeneration ability could be attributed to the altered level of endogenous hormones, variations in the degree of differentiation and their response to exogenous growth regulators in the medium. Variations in regeneration frequencies among the explants may presumably be due to predisposition of tissues from some organs to more rapid cell divisions than others and the fact that even closely associated tissues from one organ have different potentials. Excised shoots of *J. curcas* were rooted on a wide range of auxins tested, as well as in the growth regulator free MS and ½ MS medium (Table 3). Agar-gelled, full-strength MS and ½ MS medium were found to be the best for rooting (Sujatha and Mukta 1996). This may be due to the higher level of endogenous auxins in *Jatropha curcas.* Basal MS medium along with IBA (2.46µM and 4.92µM) and 2,4-D (2.26µM and 4.56µM) produced good (80%) root induction.

Significant differences were observed in the callus response, and shoot and root regeneration frequencies among all the explants. Petiole showed higher shoot regeneration frequency (50–85%) as compared to apical bud (50–75%). However, in case of leaf explants, regeneration could not be observed. Shoots were formed much earlier in direct regeneration study than roots. Shoots thus formed earlier due to reserve carbohydrates, start producing auxins which move downward and accumulate in the lower portion of the plantlets. When the concentration attains a threshold level, endogenous auxins at the extreme basal end start getting metabolized and signals the process of root initiation (Kochhar *et al.* 2005).



A. Callus induction **B. Shoot initiation**

C. Multiple shoot formation **D. Direct morphogenesis**

E. Regenerated plant transferred to pot (30 days old)

Fig. 1 (A-E). Different *in vitro* regeneration stages of *Jatropha curcas* L. from petiole explants

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