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Hormonal influence on adventitious or callus mediated regeneration from zygotic embryos in *Jatropha curcas*

MAYA KUMARI[1], VIKAS Y PATADE[2], MOHOMMAD ARIF[3], and ZAKWAN AHMED[4]

Defence Institute of Bio-Energy Research (DIBER), Goraparao, PO Arjunpur, Haldwani, Uttarakhand 263 139

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ABSTRACT

Jatropha curcas L. is being perceived as potential biofuel crop with the growing petroleum prices and depleting oil resources. Limited research has been carried out for genetic improvement in this crop. For gene enrichment through genetic manipulation an efficient, high frequency and reproducible *in vitro* regeneration protocol from any explant type is a prerequisite. In view of this, the present study was conducted to optimize *in vitro* regeneration from zygotic embryos of *Jatropha curcas*. Adventitious regeneration was obtained directly from zygotic embryos in Murashige and Skoog (MS) medium supplemented with 2 mg/l 6-benzylamino purine (BAP) (>14 shoot buds) and in 4 mg/l BAP (12 shoot buds) without addition of any other hormone. Cytokinin (BAP) combined with auxins (IAA-Indole-3-acetic acid, IBA-Indole-3-butyric acid, NAA-Naphthalene acetic acid) gave rise to callus, which continued to proliferate with maximum of 7 shoots, when transferred in regeneration media (MS + 2 mg/l BAP + 1 mg/l GA_3). This indicates the influence of hormones on the direct and indirect regeneration from the same explant. Mother explant continued to produce shoots upon successive subculturing. The highest frequency of root induction (80%) was observed on half MS medium with 0.5 mg/l IBA. Regenerated plantlets were hardened under culture room growth conditions. The present regeneration protocol may prove to be promising for genetic improvement through genetic transformation and induction of somaclonal variations through *in vitro* mutagenesis.

Key words: Adventitious regeneration, Biofuel crop, Hormones, *Jatropha curcas,* Zygotic embryos

The rising global oil prices and environmental concern has created an interest worldwide in finding new and renewable energy source for sustainable biofuel demand. *Jatropha curcas* L. is a promising green renewable energy source, known for its seed oil, yielding biofuel, having properties of fossil fuel (Ricci *et al.* 2012). *Jatropha curcas* L. commonly known as physic nut belongs to the family Euphorbiaceae, with more than 175 species distributed in tropics and sub-tropics of the world (Heller 1996). All plant parts of *Jatropha* are useful as its biomass can be used directly for generation of biofuel through pyrolysis and also by degradation of lignocellulosic waste (Openshaw 2000). It is most valued for its seed oil as a commercial source of biofuel to solve the energy crisis, reducing carbon emission and increasing the income of farmers (Zhou *et al.* 2006). Inspite of being hardy and tolerant to certain biotic and abiotic stress, its yield is limited by abiotic stress like cold.

Jatropha curcas is a monoecious, predominantly outcrossing species but is fully self-compatible and subject to geitonogamy (Rosado *et al.* 2011). Conventional propagation through seeds and cuttings is limited by problems associated with poor seed viability, low germination, and season bound availability of stem cuttings (Openshaw 2000). Also the available genetic variability is very low in *Jatropha* accession worldwide (Basha and Sujata 2007, Sudheer *et al.* 2010, Rosado *et al.* 2011) except for its center of origin. To overcome these constraints, tissue culture techniques offer continuous supply of the planting materials and also amicable for genetic manipulation for useful traits from any explant source. Hormones play a vital role in deciding the path for regeneration which may be direct through adventitious shoot buds, somatic embryogenesis or via callus. There are reports on adventitious regeneration in leaf, cotyledon, epicotyl and petiole explants of *Jatropha* (Deore and Johnson 2008, Kumar *et al.* 2010, 2011, Khemkladngoen *et al.* 2011). However, there is no report of adventitious regeneration from zygotic embryos of *Jatropha curcas*. Therefore, the present study was carried out with the aim to induce high frequency adventitious regeneration from zygotic embryos in *Jatropha curcas*.

[1] Scientist C (e mail: madhulogin@gmail.com), [2] Scientist B (e mail: vikaspatade1981@gmail.com), [3] Former Scientist E (e mail: arif527@rediffmail.com), [4] Director (Scientist G) (e mail: director@darl.drdo.in)

MATERIALS AND METHODS

Jatropha curcas germplasm DARL-2, a high oil yielding strain (36.5%) developed by DIBER, DRDO and identified at national level by Ministry of New and Renewable Energy (MNRE), Government of India, as a promising strain was used as explant source. Seeds were soaked overnight (in 0.1% bavistin) with 5% tween-20 in water, thoroughly washed under running tap and seed coat was removed without damaging the embryos. Under laminar air flow cabinet the embryos were removed from the seed and surface sterilized by treating with 70% ethanol and 0.1% (w/v) solution of $HgCl_2$ each for 30 seconds and 1 min respectively with intermittent 3 washes with sterile distilled water. Sterilized embryos were cultured on MS medium (Murashige and Skoog 1962) supplemented with 1-5 mg/l 6-benzyl amino purine (BAP) alone and also with different concentrations of auxins [Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) or Naphthalene acetic acid (NAA)] in dark for 1 week and then exposed to light once they started greening. Green elongated embryos were subsequently cultured on same medium for regeneration. The pH of medium was adjusted to 5.8 before autoclaving. Culture plates were kept inside culture room at 25±1 ºC in a 16 hr light/8 hr dark cycle.

After 4 weeks of culture, regenerated shoots were excised and cultured on MS medium supplemented with BAP and different concentrations of gibberellic acid (GA_3) for shoot elongation. For root induction, shoots (3-4 cm) were cultured on half MS basal medium supplemented with 3% (w/v) sucrose and different concentrations (0.5-2 mg/l) of auxins (IAA, IBA or NAA) for 3 weeks. The rooted plantlets were transferred to pots containing a mixture of soil and vermiculite in the ratio of 1:1 and covered with polyethylene bags inside culture room at 25±1 ºC in a 16 hr light/8 hr dark cycle for 30 days, then transferred into green house. Data on percent greening of embryos, percent regeneration, days to regeneration, number of shoots obtained, and rooting were recorded. Each treatment had five replicates with 10 explants each. The analysis was done using the program CropStat for Windows (7.2.2007.3 module), developed by the Biometrics unit, IRRI, Philippines. The treatment means were compared by using the LSD Test at significance level of $P \leq 0.05$.

RESULTS AND DISCUSSION

The zygotic embryo explant cultured on MS medium without phytohormone responded by greening without any signs of regeneration. Greening and elongation of embryos almost double of its normal size was observed in all the media combinations with hormones (Fig 1 a and b). There was significant effect of treatments on greening response (Table 1) and regeneration (Table 2). MS medium supplemented with NAA gave rise to rhizogenic callus that proliferated without any shoot bud induction (Fig 1c). There was significant effect of media on regeneration. Explants when cultured in MS medium with auxins and BAP gave rise

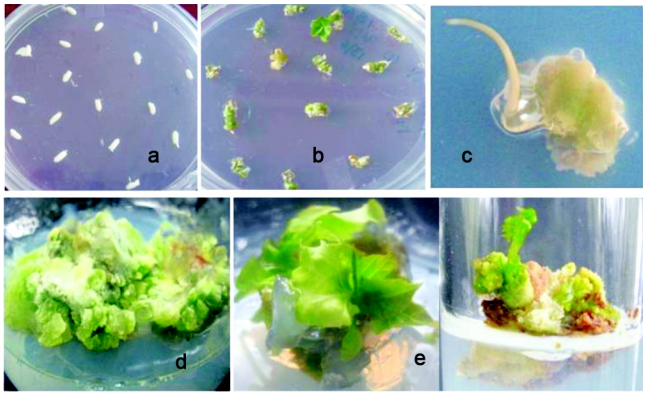

Fig 1 (a) Culture of zygotic embryos from *Jatropha curcas;* (b) Greening response of embryo in callus induction media; (c) Induction of rhizogenic calli from embryo in MS medium supplemented with 0.5 mg/l NAA; (d) Callusing and (e) regeneration in MS medium containing 1.5 mg/l BAP and 0.5 mg/l IAA and in MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l IBA.

to callus which continued to proliferate in the same medium (Fig 1d). The responding embryos showed the development of both shoot buds and callus at the base of explant cultured on MS medium supplemented with cytokinin and auxins (Fig 1e). Regeneration percentage via callus ranged from 20 to 70% with few shoots (Table 2). BAP along with IBA has been reported to increase the number of shoots in various members of Euphorbiaceae (Shrivastava and Banerjee 2008, Qin *et al.* 2004, Shah *et al.* 2010). Therefore, different concentrations of auxins along with BAP were tried for regeneration.

MS media supplemented with BAP without any other

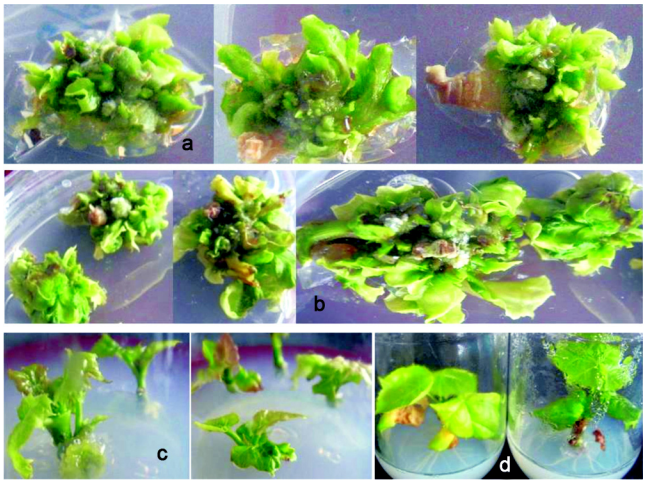

Fig 2 Adventitious regeneration into multiple shoot buds in *Jatropha curcas* embryos in (a) MS medium supplemented with 2 mg/l BAP, (b) MS supplemented with 4 mg/l BAP, (c) Elongation of shoots in MS medium supplemented with 2 mg/l BAP and 2 mg/l GA_3, (d) Rooting in half MS medium containing 0.5 mg/l IBA.

Table 1 Greening response of zygotic embryos after two weeks of culture in different treatments

MS Media + PGR (mg/l)	Greening response (%)
1 BAP	20.00[d]
2 BAP	26.67[c,d]
3 BAP	63.33[a]
4 BAP	50.00[a]
5 BAP	30.00[b,c,d]
5 BAP + 0.5 IBA	63.34[a]
3 BAP + 3 IAA	30.00[b,c,d]
3 GA_3 + 1 IAA	40.00[b,c,d]
1.5 BAP + 0.5 NAA	43.33[b]
1 NAA	13.32[d]
0.5 NAA	10.00[d]
SE	7.33
LSD (P=0.05)	21.63

Means superscripted by different alphabets in each column were significantly different at P = 0.05 according to LSD test.

Table 2 Effect of different combinations and concentrations of plant growth regulators on regeneration response from zygotic embryos of *Jatropha curcas*

MS Media + PGR (mg/l)	Percent regeneration	Days to regeneration	No. of shoot buds
Callus mediated regeneration			
BAP+0.5 IBA	20.0[d]	26.0[b]	3.0[c]
BAP+0.5 IBA	66.7[b]	20.3[d]	6.8[a]
BAP+0.5 IBA	46.7[c]	23.0[c]	5.0[b]
BAP+0.5 IBA	70.0[a]	19.0[d]	7.7[a]
BAP+0.5 IBA	60.0[a]	21.0[c,d]	5.3[b]
1.5 BAP + 0.5 IAA	43.3[c]	22.0[c,d]	3.7[c]
1.5 BAP + 0.5 IBA	25.0[d]	30.8[a]	3.9[d]
1.5 BAP + 0.5 NAA	19.1[d]	31.0[a]	2.6[d]
SE	5.09	0.88	0.62
Adventitious regeneration			
BAP	20.2[c]	39.0[a]	4.3[d]
BAP	78.0[a]	34.1[b,c]	14.7[a]
BAP	46.7[b]	36.2[a,c]	7.3[c]
BAP	76.4[a]	32.3[b]	12.2[b]
BAP	48.0[b]	34.5[b,c]	6.0[c,d]
SE	5.3	1.3	1.2

Means superscripted by different alphabets in each column were significantly different at P = 0.05 according to LSD test.

hormone, gave rise to adventitious buds (Fig 2a and b). There was significant effect of different BAP concentrations upon adventitious regeneration frequency. The highest regeneration percent was observed in MS medium supplemented with 2 mg/l BAP followed by 4 mg/l BAP without auxins giving a maximum of 14.7 and 12.2 shoots respectively in single culture (Table 2) and more than 20 shoots upon subsequent subculturing. Due to higher number of shoot buds from one shoot clump, the regenerated shoots were excised and transferred in elongation media. This allowed the remaining shoot buds to proliferate, resulting in more number of shoots per explant. This may be because of internal competition between shoot buds for nutrients. Thus the mother explant can be maintained by continuous subculturing in order to obtain continuous supply of shoots (Shah *et al.* 2010).

Table 3 Elongation response of regenerated shoots in different elongation medium.

MS Media + PGR (mg/l)	Percent elongation	Days to elongation	Shoot length (cm)
1 GA_3	13.3[d]	39.0[a]	2.7[c]
1.5 GA_3	20.0[d]	36.3[a]	2.6[c]
2 GA_3	23.3[c]	35.0[b]	3.5[a]
1 GA_3 + 2 BAP	36.7[b]	31.7[b]	3.0[b]
1.5 GA_3 + 2 BAP	46.7[b]	31.3[c]	3.4[a]
2 GA_3 + 2 BAP	60.0[a]	29.0[c]	3.9[a]
SE	4.2	1.1	0.3

Means superscripted by different alphabets in each column were significantly different at P = 0.05 according to LSD test.

Different concentrations of GA_3 (0.5–2 mg/l) along with BAP (2 mg/l) were tried for shoot elongation (Table 3). There was significant difference in elongation response with GA_3 concentrations. Elongation was best (60%) in MS supplemented with 2 mg/l BAP and 2 mg/l GA_3 taking least number of days (29 days) and giving maximum (3.9 cm) shoot length (Table 3). On an average 2.5 cm shoot length was observed within 4 weeks of sub-culturing on elongation media (Fig 2c). The promoting effect of GA_3 on elongation of stunted shoots, generated on BAP containing medium, has been reported in other plant species (Shah *et al.* 2010). Elongated shoots when cultured on MS medium supplemented with various concentrations of auxins gave significant difference for root induction (Fig 2d, Table 4). Half MS with auxins (IAA and IBA) gave better rooting response compared to full MS without auxins and MS with NAA. MS media supplemented with 0.5 mg/l IBA was observed best for rooting

Table 4 Rooting response of elongated shoots in different rooting treatments

MS Media + PGR (mg/l)	Percent rooting	Days to rooting
Full MS	21.0[c]	21.0[a]
Half MS	33.0[b]	16.3[b]
Half MS+ 0.5 mg/l IAA	50.0[a]	14.3[b]
Half MS+ 0.5 mg/l IBA	66.8[a]	16.7[b]
Half MS+ 0.5 mg/l NAA	30.0[b]	20.3[a]
SE	5.4	0.8

Means superscripted by different alphabets in each column were significantly different at P = 0.05 according to LSD test.

giving higher number of roots (5) and root length (8 cm). IBA has been reported for best rooting response in *J. curcas* (Datta *et al.* 2007, Shrivastava and Banerjee 2008) compared to IAA and NAA. Soil and vermiculite (1:1) mixture was used for hardening. Regenerated plantlets showed normal growth and development after hardening without any phenotypic abnormalities.

CONCLUSION

There are few reports describing protocols for adventitious regeneration from various explant types such as cotyledons, leaves, petioles and hypocotyls of *J. curcas* (Datta *et al.* 2007, Deore and Johnson 2008, Kumar *et al.* 2010, 2011, Khemkladngoen *et al.* 2011). In this study we report high frequency regeneration system from zygotic embryos (~80%) of *Jatropha curcas*, which is the first report on adventitious regeneration from zygotic embryos of *Jatropha*. The protocol developed here is simple, provides high regeneration frequency, and uses low cost hormones in lower concentrations (2-4 mg/l BAP, 2 mg/l GA_3, and 0.5 mg/l IBA). The protocol reported in this study can be used for genetic manipulation and clonal selection via *in vitro* mutagenesis, to induce genetic variation in the existing genepool of *Jatropha*.

REFERENCES

Basha S D and Sujatha M. 2007. Inter and intra-population variability of *Jatropha curcas* (L.) characterized by RAPD and ISSR markers and development of population-specific SCAR markers. *Euphytica* **156**: 375–86.

Datta M M, Mukherjee P, Ghosh B and Becker K. 2007. *In vitro* clonal propagation of biodiesel plant (*Jatropha curcas* L.). *Current Science* **39**: 1 438–42.

Deore A C and Johnson T S. 2008. High-frequency plant regeneration from leaf-disc cultures of *Jatropha curcas* L.: an important biodiesel plant. *Plant Biotechnology Reports* **2**: 7–11.

Heller J. 1996. Physic nut, *Jatropha curcas*. Promoting the conservation and use of underutilized and neglected crops 1. IPGRI, Rome, Italy, 1–66. http://www.bioversityinternational.org/publications/publications/publication/issue/physic_nut_brijatropha_curcasi_l.html.

Khemkladngoen N, Cartagena J, Shibagaki N and Fukui K. 2011. Adventitious shoot regeneration from juvenile cotyledons of a biodiesel producing plant, *Jatropha curcas* L. *Journal of Bioscience and Bioengineering* **3**: 67–70.

Kumar N, Anand K G V and Reddy M P. 2010. Shoot regeneration from cotyledonary leaf explants of *Jatropha curcas*: a biodiesel plant. *Acta Physiologia Plantarum* **32**: 917–24.

Kumar N, Anand K G V and Reddy M P. 2011. *In vitro* regeneration from petiole explants of non-toxic *Jatropha curcas*. *Industrial Crops and Products* **33**: 146–51.

Murashige T and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473–9.

Openshaw K. 2000. A review of *Jatropha Curcas* L: an oil plant of unfulfilled promise. *Biomass Bioenergy* **19**: 1–15.

Qin W, Wei-Da L, Yi L, Shu-Lin P, Ying X U, Lin T and Fang C. 2004. Plant regeneration from epicotyl explant of *Jatropha curcas*. *Journal of Plant Physiology and Molecular Biology* **30**(4): 475–8.

Ricci A, Chekhovskiy K, Azhaguvel P, Albertini E, Falcinelli M and Saha M. 2012. Molecular characterization of *Jatropha curcas* resources and identification of population-specific markers. *Bioenergy Research* **5**: 215–24.

Rosado T B, Laviola B G, Faria D A, Pappas M R, Bhering L L, Quirino B and Grattapaglia D. 2010. Molecular markers reveal limited genetic diversity in a large germplasm collection of the biofuel crop *Jatropha curcas* L. in Brazil. *Crop Science* **50**: 2 372–82.

Rug M and Ruppel A. 2000. Toxic activities of the plant *Jatropha curcas* against intermediate snail hosts and larvae of schistosomes. *Tropical Medicine and International Health* **5**: 423–30.

Shah P, Khare N, Oreya S, Arif M and Ahmed Z. 2010. *In vitro* plant regeneration from microshoot in *Jatropha curcas*. *International Journal of Agriculture and Food Science and Technology* **1**(1): 63–72.

Shrivastava S and Banerjee M. 2008. *In vitro* clonal propagation of physic nut (*Jatropha curcas* L.): Influence of additives. *International Journal of Integrative Biology* **3**(1): 73–9.

Sudheer P D V N, Mastan S G, Rahman H and Reddy M P. 2010. Molecular characterization and genetic diversity analysis of *Jatropha curcas* L. in India using RAPD and AFLP analysis. *Molecular Biology Reports* **37**(5): 2 249–57.

Zhou H, Lu H and Liang B. 2006. Solubility of multicomponent systems in the biodiesel production by transesterification of *Jatropha curcas* L. oil with methanol. *Journal of Chemical Engineering Data* **51**: 1 130–5.