Int J Pharm Bio Sci 2013 Jan; 4(1): (B) 673 - 682

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

EFFICIENT METHOD FOR DIRECT AND INDIRECT ORGANOGENESIS IN BIOFUEL CROP JATROPHA CURCAS

PURNA SHUKLA[1], VIJAY MAKWANA[1], DAKSHA BHATT[2], AND PUSHPA ROBIN[1]*

[1]Department of Biochemistry, The Maharaja Sayajirao University of Baroda, Vadodara 390 002, India [2]Sun Agrigenetics Ltd. Vadodara 390 007, India

ABSTRACT

Protocol for high frequency regenerants of *J. curcas* has been developed by the process of direct and indirect organogenesis from nodes and leaves as explants. Both the explants were initially inoculated on Murashige and Skoogs (MS) Basal medium. After a week they were transferred to MS medium supplemented with combinations of 6-benzylaminopurine (BAP) and Indole-3- butyric acid (IBA). MS medium supplemented with 3.0 and 5.0 µM IBA with 27.0 µM BAP supported quick callus initiation, while increasing the concentration of IBA to 7.5 µM led to delay in callusing. Emergence of shoot bud was first observed on medium supplemented with 27.0 µM BAP with 3.0 µM IBA. Of the combinations reported here 3.0 µM IBA with 4.5 µM and 27.0 µM BAP was found to be the best suitable medium for promoting multiple shoot regeneration with offshoot measuring 1.5-2.0 cm. Rooting was observed on MS basal medium.

KEYWORDS: **BAP (6-benzylaminopurine); IBA (Indole-3-butyric acid); callus; shoot bud initiation; shoot regeneration; *Jatropha curcas***

PUSHPA ROBIN
Department of Biochemistry, The Maharaja Sayajirao University of Baroda, Vadodara 390 002, India

Corresponding author

INTRODUCTION

Jatropha curcas is a drought resistant shrub belonging to the genus Euphorbiaceae. It has been touted as a biodiesel plant. Today, *Jatropha curcas* is mainly cultivated as a biofuel crop on marginal land for poverty alleviation. *Jatropha* has also been exploited for its medicinal properties[1]. Commercial success of *Jatropha curcas* as a biofuel plant remains elusive despite earnest efforts by both scientists and agriculturists. Major bottlenecks for these limitations are significant variations in seed yield and oil content, low seed viability and germination rate. For vegetative propagation to be successful, it would require availability of quality planting material throughout the year. However, this is limited due to unavailability of quality planting material and propagation being affected by climatic changes. Improvement in *J. curcas* potential for biofuel production by modern methods of agro-biotechnology is of interest worldwide. For this development of tissue culture protocols to facilitate large scale production of true-to-type plants is important. These protocols could also be used for the improvement of the species by genetic engineering techniques. In our laboratory too we have demonstrated increased salt tolerance in groundnut by genetic transformation[2]. *In vitro* regeneration techniques offer a powerful tool for germplasm conservation, mass multiplication of true-to-type plants and genetic transformation[2]. Genetic transformation approach allows introduction of novel genes thereby helping in introducing or improving agronomically important traits. The availability of an efficient regeneration system is a prerequisite for utilizing this approach. For successful genetic modification, efficient production of transgenic plants, effective and fast regeneration system is imperative. Few regeneration protocols have been reported for *J. curcas* using different explants like leaves [4,5,6.7.8,9]; shoot tips [10]; nodes and axillary nodes [5]; petiole [3,4,11]; hypocotyls[4]; cotyledons[12] etc. Both direct and indirect plant regeneration systems have been reported. Though several reports on regeneration from various explants in *J. curcas* exist, not many reports on its genetic transformation are available. This is due to the lack of efficient protocols to regenerate whole plants through *in vitro* regeneration from the transformed tissue. Here we report an efficient method for regeneration of *J. curcas.*

MATERIALS AND METHODS

(i) Plant Material and general culture conditions

Jatropha curcas plants grown on The M.S. University Campus were used as source of explants. Tissue culture media were prepared as per specifications in Murashige and Skoog (MS) (1962) having 30g/L sucrose. The pH of the media was adjusted to 5.6 ± 0.1 using 1N NaOH or 1N HCl and supplemented with 7g/L agar. Media was autoclaved at 15 psi at 121ºC for 20 minutes. Growth conditions were maintained at 26±2°C with 16 h photoperiod with flux density of 30 µmol m^{-2} s^{-1} by cool white fluorescent tubes (Philips, India).

(ii) Jatropha curcas explants

For leaf explants young leaves at 3rd and 4th node from the apex were collected from 1.5-2 year old plants. For experiments on culture initiation from axillary buds, tender, thin, green twigs cut below 2nd and 3rd nodes were taken as explants. Each explant would contain one apical bud and 2 to 3 axillary buds. The explants were thoroughly washed with tap water for nearly half an hour followed by washing with soap solution. The explants were surface sterilized with 0.1% mercuric chloride for 2 to 3 minutes (leaf explants) and 6 minutes (nodal explants) followed by five rinses with sterile distilled water. Leaves were then excised into small pieces of 1x1cm and

nodes were trimmed to 1.0-1.5 cm and inoculated on Murashige & Skoog (MS) basal medium. After a week these explants were transferred to media containing different combination of BAP and IBA for regeneration. They were observed periodically and their properties were recorded. A week after inoculation on MS basal medium, the leaf explants were cultured on (4.5 -27.0 µM) BAP and (3.50 -7.5 µM) IBA whereas nodes were cultured on MS supplemented with 2.2 µM BAP and 4.9 µM IBA. Nodes were further sub-cultured on MS medium supplemented with 8.9 µM BAP and 2.9 µM IAA with adenine sulphate (100mg/l) and glutamine (100 mg/l) as an addendum. The cultures were incubated at 26 ± 2 ºC under a 16 h photoperiod using cool, white fluorescent light (30 µmol m^{-2} s^{-1}).

(iii) Data Analysis

Experiments were set in completely randomized design. All the experiments were repeated thrice and had ten replicates with single explants. Observations on number of explants forming callus, bud initiation and number of explants forming multiple shoots per explants were recorded. All data obtained were subjected to analysis of variance and significant differences in values were calculated according to Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Indirect organogenesis through leaf discs

In this study, adventitious shoot regeneration from leaf explants was attempted. It is a known that a balance between auxin and cytokinin normally induces effective organogenesis. Though the nature of interaction between the two plant growth regulators is not completely understood, cell division seems to be regulated by their interactions affecting different phases of cell cycle. While auxins are known to exert an effect on DNA replication, cytokinin exerts some control over the events leading to mitosis[14]. Therefore normal cell divisions would require synchrony between S phase and cell division suggesting that auxin and cytokinin levels in culture be carefully matched. Reports have shown the importance of BAP and IBA in inducing organogenesis from leaf discs [4,5,10]. In the current investigation a range of combination of BAP (4.5 - 27.0 µM) and IBA (3.0 -7.5 µM) were used for indirect organogenesis. Curling of enlarged leaf discs was first observed followed by callus appearance at the cut margins (Figure 1).

Figure1
Callus initiation at the cut margin

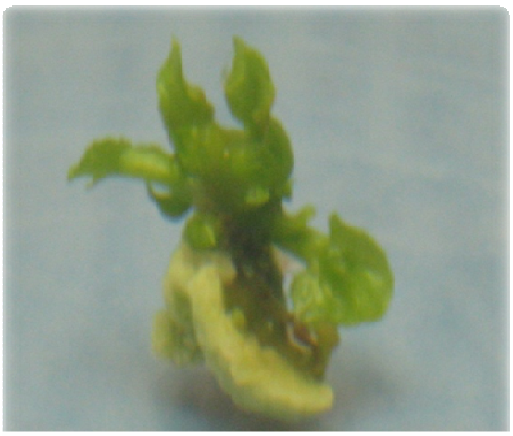

Figure 2
Emerging shoot buds from callus

No significant difference was observed in callusing and regeneration frequency from the explants derived from 3rd and 4th expanding leaf. This is in contrast to report by Sujatha and Mukta, (1996) [4] who reported a differential response for the two. Callus morphology and relative response of callusing was studied. In MS media supplemented with both BAP and IBA at highest levels i.e. 27.0 µM and 7.5 µM respectively, callus formation was very less and much slower as compared to other combinations. BAP levels as low as 9.0 µM with 5.0 µM IBA also failed to induce sufficient callus (data not shown). Ex-plants on MS medium supplemented with lower levels of IBA (3.0 µM and 5.0 µM) and higher levels of BAP gave maximum callus formation. Similar results were obtained in MS fortified with high levels of IBA in combination with low BAP. Though MS media containing highest levels of either BAP or IBA showed massive callus formation no organogenesis was observed. Even on sub-culturing on respective media it continued to form callus. In the medium supplemented with 13.0 µM BAP + 7.5 µM IBA and 27.0 µM BAP + 7.5 µM IBA, callus turned brown and dried after first passage. By reducing IBA to 3.0 µM in combination with all three concentrations of BAP, organogenesis was observed after 45 days in culture. In the present study, tweaking cytokinin levels did not have an effect on organogenesis while low IBA levels did influence organogenesis. Low levels of IBA play a key role in callus formation, bud initiation and multiple shoot formation. This is substantiated by Kalimuthu et al, (2007)[15] where high concentrations of auxins were shown to be inhibitory to morphogenesis and the use of an appropriate auxin-cytokinin ratio is essential to obtain proper shoots and root primordia. This could be suggestive of the importance of lower levels of IBA in inducing organogenesis. Buds in clusters (3-5) started emerging from various locations of the callus, mostly underneath the explants (Figure 2). First bud initiation was observed in MS medium supplemented with 27.0 µM BAP + 3.0 µM IBA. It was also the most effective combination for shoot bud initiation and proliferation (Table 1, Figure 3). In the current study, MS+27.0 µM BAP + 3.0 µM IBA resulted in 10 buds per regenerating calli. Out of these 9.33 buds showed proliferation on the same combination. Khurana-Kaul et al, (2010) [8] reported 8.6 buds in MS+13.33 µM BAP + 2.46 µM IBA. Using similar combination also gave rise to 8.0 buds per regenerating calli in the present study. Khurana-Kaul et al, (2010) [8] have also shown the influence of Thiaduzaron (TDZ) in achieving higher number of shoot buds.

Figure 3
Shoot bud initiation and proliferation

Table 1
Effect of BAP and IBA on formation of buds

BAP/IBA (µM)	Number of buds per explants
	After 6 weeks
27.0/3.0	10 ± 2.35 [a]
13.0/3.0	8.0 ± 1.41 [a]
4.5/3.0	5.0 ± 0.94 [b]
27.0/5.0	2.0 ± 0.94 [c]
4.5/7.5	0.0 ± 0.0 [c,d]

Means in each column followed by same letters are not significantly different according to DMRT at α=0.05 at 12 degrees of freedom.

While in the present study results have been achieved even in the absence of TDZ. Sujatha and Mukta, (1996) [4] reported 2.22 µM BAP and 2.46 µM IBA to give 10.7 shoots per regenerating calli. A few other media combinations in the present study showed the same response a little later. As shown in table-2, there is a significant difference between all the media combinations studied except for the two containing isomolar concentration of IBA (3.0 µM) with varying BAP levels (13.0 µM and 27.0 µM). This indicates that the increased level of cytokinin fails to show its impact on initiation of organogenesis. However, after organogenesis has been successfully induced and shoot bud proliferation is observed, increased IBA levels have little influence (Table 2).

Table 2
Effect of BAP and IBA on proliferation of buds

BAP/IBA (µM)	Number of buds per explants
	After 10 weeks
27.0/3.0	9.33 ± 0.27 [a]
27.0/5.0	8.66 ± 0.54 [a]
13.0/3.0	6.00 ± 1.63 [b]
4.5/3.0	5.33 ± 1.90 [c]
4.5/7.5	0.0 ± 0.0 [d]

Means in each column followed by same letters are not significantly different according to DMRT at α=0.05 at 12 degrees of freedom

Of the four media combinations used for formation of new buds, MS medium supplemented with 13.0 µM BAP +3.0 µM IBA did not show further response. However, other media combinations continued to show their effect on the number of shoot regenerants. Table-3 depicts the mean length of regenerants with shoot length of more than 1.0 cm. In some explants, regenerants of more than 1.5-2.0 cm were seen (Figure 4).

Table 3
Effect of BAP and IBA on shoot length of bud regenerants

BAP/IBA (µM)	Mean shoot length	% frequency
4.5/3.0	>1.0cm	62.5
27.0/3.0	>1.0cm	55.5
27.0/5.0	>1.0cm	0

Shoot length of regenerants measuring more than 1.0 cm only have been considered.

Figure 4
Regenerants achieved from callus

The MS medium supplemented with 4.5 µM BAP + 3.0 µM IBA and 27.0 µM BAP + 3.0 µM IBA are not significantly different according to DMRT (α=0.05). Kumar et al, (2010)[12] achieved elongation on MS+ 4.5 µM BAP + 7.5 µM IBA with shoot length of 2.3-2.5cm. The current investigation reports an effective combination for successful regeneration of shoots from leaf discs derived calli. It could be concluded that auxins play a greater role than cytokinins in organogenesis of *J. curcas* while changes in cytokinin levels did not yield significant changes in shoot bud induction, a small increment in auxin levels gave rise to appreciable changes in the same. Hence, it could be concluded that irrespective of BAP levels, low levels of IBA are essential in order to obtain multiple shoot regenerants per explant. Deore and Johnson, (2008) [4]have shown the synergistic role of thiadiazuron (TDZ) and BA in inducing shoots. They have also shown the reduced effect of BA in absence of TDZ. In the present study MS supplemented with 4.5 µM BAP + 3.0 µM IBA and 27.0 µM BAP + 3.0 µM IBA showed 100% frequency for callus formation, shoot bud induction and proliferation. Shoot elongation was studied in MS+8.8 µM BAP+ 2.5 µM IAA, MS+8.8 µM BAP+ 2.5 µM IAA+1.0 µM Kn, MS+22.0 µM BAP+ 2.5 µM IAA+1.0 µM Kn and MS+8.8 µM BAP+ 2.5 µM IAA+5.0 µM Kn. Out of these combinations studied, sprouting of new shoots was observed in MS+8.8 µM BAP+2.5 µM IAA+1.0 µM Kn and MS+22.0 µM BAP+ 2.5 µM IAA+1.0 µM Kn. MS+8.8 µM BAP+ 2.5 µM IAA+1.0 µM Kn also favored quick and effective growth of existing shoots along with MS+8.8 µM BAP+ 2.5 µM IAA+5.0 µM Kn. It is a widely known fact that root formation in *in vitro* grown plants is triggered or induced by external application of auxins. In the work reported on *J. curcas* MS in conjunction with NAA [5] and IBA [6], respectively, has been used for root formation. In the present study, rooting was observed after three weeks of transfer in the rooting medium (Figure 5). Various media combinations were studied to induce rooting in regenerated shoots that had achieved length of more than 1.5-2.0 cm. The media combinations used to study rooting were MSB +11.0 µM NAA, MSB +21.0 µM NAA, MSB +50.0 µM NAA, Half strength MS+9.8 µM IBA+2.22 µM BAP, MS basal medium and direct transfer of the explants to vermiculite. Rooting could be achieved on MS basal medium. This is in contrast to the studies reported by Sujatha and Mukta, (1996) [4] wherein rooting was observed on MS basal medium within 8-10 days with 88% rooting frequency. However, in the present investigation, more time was taken for root formation.

Figure 5
Root formation

Direct organogenesis in J. curcas

Direct shoot regeneration has been reported from a number of explants like shoot tip, axillary node, petiole, leaf disc, immature embryo, cotyledonary leaf, epicotyls, hypocotyls etc. of toxic and non-toxic varieties of *J. curcas* ([3,5,6,10,15,17,18]). Of all these reports, only a few have reported direct organogenesis from shoot tips, nodes or axillary buds as explants ([5.10,15]). Direct plant regeneration without an intervening callus phase is a more reliable method for multiplication or clonal propagation. The plants produced by direct organogenesis may exhibit greater genetic stability than those produced via callus mediated organogenesis ([19]). Murashige and Skoog's ([13]) medium is the reported media for all the above mentioned studies on *J. curcas*. Plant growth regulators used for direct regeneration include TDZ, BA, Kn, IBA and IAA. These growth regulators were used at different levels either singly or in combination. In the present study for experiments on culture initiation from axillary buds, tender, thin, green twigs cut below 2nd and 3rd nodes were taken as explants. Each explant containing one apical bud and 2 to 3 axillary buds were pretreated and surface sterilized with mercuric chloride. The culture medium comprised of MS medium supplemented with 2.2 µM BAP and 4.9 µM IBA. Nodal explants produced single shoots within 10-12 days (Figure 6). Similar results have been reported by Kalimuthu et al[15] where MS basal medium was used for culture initiation. Further multiplication of shoots was achieved in MS fortified with BAP, Kn and IAA. Sujatha et al, (2005) [5] reported the use of BA, Kn and TDZ. Except for one concentration of Kinetin, none of the combination with the same showed any visible signs of explants differentiation except for bud emergence with very low axillary proliferation. We also found that TDZ was more effective as compared to BA and Kinetin. It is a common fact that cytokinins and auxins were found essential for shoot multiplication. The higher concentration of auxins is generally inhibitory to morphogenesis and substitution of these reagents with an appropriate auxin-cytokinin ratio is essential to obtain proper shoot and root primordia ([15]). In the current investigation, explants were sub cultured on MS medium supplemented with 2.22 µM BAP and 4.92 µM IBA. Though apical bud is mostly the preferred explant for obtaining direct regeneration owing to its meristematic nature, it was found that apical bud was more prone to contamination. Multiple shoot formation was observed within a period of 30-40 days (Figure 7). Multiplication of shoots was obtained in the same combination.

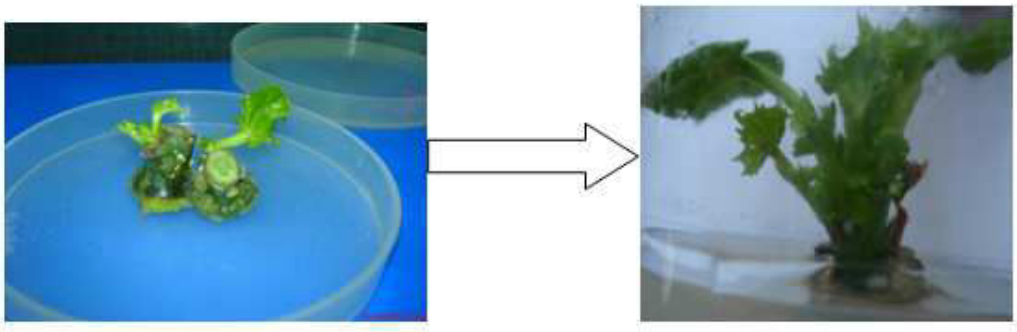

Figure 6: Emergence of new shoot bud

Figure 7: Multiplication of nodal culture

The media combinations used to study rooting were MSB +11.0 µM NAA, MSB +21.0 µM NAA, MSB +50.0 µM NAA, half strength MS +9.8 µM IBA+2.22 µM BAP, MS basal medium and direct transfer of the explants to vermiculite. The shoots were then kept on MS basal medium for rooting. Roots were observed after nearly three weeks in MS basal medium.

CONCLUSIONS

From the above studies it can be concluded that MS salts fortified with 2.2 µM BAP and 4.9 µM IBA successfully gave rise to new shoots and multiplication of shoots was also observed in the same.

CONFLICT OF INTEREST: NONE

REFERENCES

1. Henning, R.K. The *Jatropha* system-an integrated approach of rural development (available at www.*Jatropha*.de), (2004)
2. Vadawale, A., Mihani, R., Mathew, A., Robin, P. Transformation of Groundnut-Arachis Hypogea L. Var GG20 with the Cox gene-An attempt to develop salinity tolerance. International Journal of Pharma and BioSciences: 3(1): 591-599, (2012)
3. Kumar, N., Reddy, M.P. Plant regeneration through the direct induction of shoot buds from petiole explants of *Jatropha curcas*: a biofuel plant. Ann. of App. Bio, 156: 367-375, (2010)
4. Sujatha, M., Mukta, N. Morphogenesis and plant regeneration from tissue cultures of *Jatropha curcas.* Plant Cell Tiss Organ Cult, 44: 135-141(1996)
5. Sujatha, M., Makkar, H.P.S., Becker, K. Shoot bud proliferation from axillary nodes and leaf sections of non-toxic *Jatropha curcas* L. Plant Growth Reguln, 47: 83-90 (2005)
6. Deore, C.A., Johnson, S.T. High frequency plant regeneration from leaf disc cultures of *Jatropha curcas* L.: an important biodiesel plant. Plant Biotechnol Reports, 2: 7-11(2008)
7. Reddy, M.P., Kumar, N., Vijay Anand, K.G., Singh, A.H., Singh, S. Method for micropropagation of *Jatropha curcas* plants from leaf explants (Patent filed US and PCT, File No. 2537de2008
8. Khurana-Kaul, V., Kachhwaha, S., Kothari, S.L. Direct shoot regeneration from leaf explants of *Jatropha curcas* in response to thidiazuron and high copper contents in the medium. Inter J of Pure and Appld Sci and Tech, 1(1): 11-24(2010)
9. Divakara, B.N., Upadhyaya, H.D., Wani, S.P., Laxmipathi Gowda, C.L. Biology and genetic improvement of *Jatropha curcas* L.: a review. Appld Energy, 87:732-742 (2010)
10. Rajore, S., Batra, A. Efficient plant regeneration via shoot tip explants in *Jatropha curcas.* Journal of Plant Biochem and Biotech, 14:73-75 (2005)
11. Kumar, N., Vijay Anand, K.G., Reddy, M.P. In *vitro* regeneration from petiole explants of non-toxic *Jatropha curcas.* Ind. Crops and prod, 33: 146-151 (2011)
12. Kumar, N., Vijay Anand, K.G., Reddy, M.P. Shoot regeneration from cotyledonary leaf explants of *Jatropha curcas*: a biodiesel plant. Acta Physiologiae Planta, 32, 917–924, DOI 10.1007/s11738-010-0479-9 (2010)
13. Murashige, T., Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Planta, 15: 473-497(1962)
14. Pasternak, T., Miskolczi, P., Ayaydin, F., M´esz´aros, T., Dudits, D., Feh´er, A. Exogenous auxin and cytokinin dependent activation of CDKs and cell division in leaf protoplast-derived cells of alfalfa. Plant Growth Reguln, 32:129–141(2000)
15. Kalimuthu, K., Paulsamy, S., Senthilkumar, R., Sathya, M. *In vitro* propagation of the biodiesel plant *Jatropha curcas L*. Plant Tissue Culture & Biotechnology 17(2): 137-147 (2007)
16. Shrivastava, S., Banerjee, M. In *vitro* clonal propagation of physic nut (*Jatropha curcas* L.): Influence of

additives. Int. J. of Int Bio, 3(1):73-79 (2008)
17. Kumar, N., Vijay Anand, K.G., Reddy, M.P. In vitro Plant Regeneration of Non-toxic *Jatropha curcas* L.: Direct Shoot Organogenesis from Cotyledonary Petiole Explants. J. of Crop Sci and Biotechno, 13(3):189-194 (2010a)
18. Mukherjee, P., Varshney, A., Johnson, T.S., Jha T.B. *Jatropha curcas*: a review on biotechnological status and challenges. Plant Biotechnol Reports 5:197-215 (2011)