High Concentration of Thidiazuron Stimulates Adventitious Bud Regeneration from Cotyledon Explants in *Jatropha curcas*

Ying LIU [a], Hong-Bo ZHU [b], Jian-Nong LU [c], Lin-Feng LI [d], Yu-Zhen SHI [e], Xue-Gui YIN [f, *]

Faculty of Agricultural Science, Guang Dong Ocean University, Haida Road #1, Mazhang District, 524088, Zhanjiang, Guangdong, P. R. China.

[a]liuying85168@126.com, [b]39198351@qq.com, [c]2529553658@qq.com, [d]149327957@qq.com, [e]shyzh1102@163.com, [f]yinxuegui@126.com

*Corresponding author

Keywords: Jatropha curcas, Cotyledon explants, Plant regeneration, Thidiazuron.

Abstract. A high-frequency protocol for induction of adventitious buds and plant regeneration from cotyledon explant of *Jatropha curcas* is described. The cotyledon explants of *J. curcas* cultured directly onto medium containing Thidiazuron (TDZ) induced regeneration of only poor quality adventitious buds that had a low regeneration frequency, and subsequently the elongation of shoot-buds was difficult and unsatisfactory. However, treating the cotyledon explants with high concentrations (10-60 mg/L) of TDZ solution for short time periods (10-60 min) helped to improve the regeneration frequency and enhance the quality of the regenerated shoot-buds significantly. The best shoot-buds induction (87.45%) and number of shoot-buds (11.23) per explant were seen when *in vitro* explants were treated with 20 mg/L TDZ solution for 40 min before being inoculated onto hormone-free Murashige and Skoog (MS) medium in 30 days. The elongated shoots initiated roots to become intact plantlets in rooting medium supplemented with 0.1 mg/L indole-3-butyric acid (IBA) effectively stimulated the initiation and growth of roots with the best rooting rate (44.28%). After acclimatization, these plantlets were transplanted to soil wherein normal growth was observed. Hence, an intact plantlet could usually often be gained at 60 to 70 days of culture by applying the culture method described in the present study. This protocol could be widely used for mass production of regenerative plants and the yield of transgenic plants through *Agrobacterium*-mediated transformation.

Introduction

Jatropha curcas L. is the common name for physic nut and belongs to the *Euphorbiaceae* family [1]. *J. curcas* is a type of woody plant widely distributed in the tropical and sub-tropical areas [2]. The seeds and oil from *J. curcas* are unfit for human and animal consumption because of the presence of toxins such as curcin, phorbol esters and saponins [3-6]. The latex from *J. curcas* contains alkaloids such as Jatrophine and Jatropham, which are reported to have medicinal properties and can be used for extracting pharmaceutical compounds [7-8] and insecticides [9]. However, this tree is most famous for the high oil content in its seeds (up to 60%) [10], which could be easily processed to replace the conventional fossil diesel [11]. There has been a surge of interest in *J. curcas* cultivation around the world since studies showed that its methyl ester could yield biodiesel [8-13].

To meet the ever increasing demand for biodiesel, feedstock and medicinal applications, it is necessary to develop toxic-free, high-yielding, and biotic and abiotic stress resistant *J. curcas*. However, conventional breeding is hampered by low and inconstant seed yield due to heterozygous nature of *J. curcas* plants and sexual incompatibilities due to deleterious gene linkages, followed by difficulty in obtaining large seed yield, fertile progenies and requires a very long time [14,15].

Biotechnological innovations are envisaged for genetic upgrading of this crop wherein transgenic production will be the most important in achieving the above parameters.

In a genetic transformation system, regeneration of adventitious buds from the genetically transformed tissues (explants) is in most cases an essential step. To apply *Agrobacterium*-mediated genetic transformation approach to *J. curcas* improvement more rapidly and efficiently, one prerequisite is to establish an efficient plant regeneration system. Previously, shoot-buds regeneration of *J. curcas* had been successfully obtained from cotyledon, petiole, hypocotyl, epicotyl, and leaf tissue by conventional culture methods, but those regeneration efficiency were still not satisfactory and the periods of obtaining regenerated plants were too long (90-150 days) [16-23].

In this study, we have developed an efficient and reproducible plant regeneration procedure for *J. curcas* by utilizing high concentration of TDZ solution to deal with the cotyledon explants.

Materials and Methods

Plant Materials

Seeds coded M-19 of *J. curcas* were grown and collected from the farm in Haikou, Hainan province of China [27].

Preparation of Culture Medium and Culture Maintenance

Uniform culture conditions were applied in all experiments. Basal MS formula was used for all tissue culture experiments. Media used in our experiment contained 2.5% sucrose that were adjusted to pH 5.8-6.0 with 1 mol/L NaOH, and had 0.7% agar added prior to being autoclaved at 1.4 kg cm-2 for 20 minutes. All culture treatments were kept at 25 ± 1℃ under a 12 h photoperiod of 60-80 μmol m-2s-1 intensity (cool white fluorescent tubes).

Preparation of Explants

The seeds were surface-sterilized for 60 s with 75% (v/v) ethanol after removing the outer seed coat, immersed in 2% (v/v) sodium hypochlorite (NaClO) for 20 min and finally rinsed 5 times in sterile distilled water. The embryos were removed from the seed and incubated on hormone-free Murashige and Skoog salt (MS) medium [24]. Cotyledons were excised from 5-day-old seedlings, cut into small pieces (5×5 mm), and used as explants.

Preparation of Thidiazuron (TDZ) Solution and Treating the Cotyledon Explants with TDZ Solution

TDZ (Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved in 1 mol/L NaOH solution. The solution was diluted with purified water to make the following concentrations: 0, 10, 20, 30 and 60 mg/L. These were adjusted with 1 mol/L HCl or 1 mol/L NaOH to obtain a pH value range of 5.8-6.0, and filter-sterilized. Cotyledon explants were soaked in glass bottles containing different concentrations (0, 10, 20, 30 and 60 mg/L) of TDZ solution for various time periods (0, 10, 20, 40 and 60 min). After treatment, the explants were briefly placed on sterile dry filter paper in petri dish to absorb excess moisture.

Direct Induction of Adventitious Shoots Regeneration and Elongation of Shoots Using Cotyledon Explants of J. curcas

For inducing shoot-buds regeneration, cotyledon explants were inoculated on hormone-free MS medium after treated with TDZ solution for various time periods. For comparison, cotyledon explants were also treated using conventional methods and inoculated directly on MS medium containing different concentrations of TDZ (0, 0.3, 0.6 and 1.2 mg/L) as reported previously [20-22]. The percentage of induction of shoot buds and the number of shoot buds per explant were recorded after 30 days of culture. For shoot buds elongation, the regenerated shoot buds were transferred along with the mother tissues (explants) to MS medium supplemented with 0.4 mg/L gibberellic acid (GA3), 0.5 mg/L 6-benzylaminopurine (BA), 0.2 mg/L kinetin (KT) and 0.25 mg/L indole-3-acetic acid (IAA)

(Sigma-Aldrich Co., St. Louis, MO, USA) [23,27]. The length of the elongated shoots was recorded after 15 days of culture.

Rooting Culture

For inducing rooting of the elongated shoots, shoots at least 1 cm in length were isolated from the mother tissues and inoculated onto fresh half-strength MS (1/2 MS) medium supplemented with different concentrations (0, 0.1, 0.3, 0.6 and 1.2 mg/L) of indole butyric acid (IBA) (Sigma-Aldrich Co., St. Louis, MO, USA), and the results were assessed on 10, 20, 30 and 40 days of culture.

Evaluation of the Results and Data Analysis

All experiments were set up in a completely randomized factorial design and repeated three times with 25-30 replicates per treatment. Statistical analysis of the data was carried out using SPSS 17.0 soft ware, and data in the same column followed by different letters were significantly different at $p \leqslant 5\%$ level as determined by Duncan's multiple range test. The results were expressed as means ± SD (standard deviation) of three independent experiments.

Results

Regeneration and Elongation of Adventitious Buds from Cotyledon Explants with Conventional Culture Methods

Cotyledon explants without TDZ solution treatment were inoculated onto MS medium containing different concentrations of TDZ as reported previously [20-22]. The concentration of TDZ in the medium influenced the response of shoot buds induction for tested (Table 1). Of the different concentrations of TDZ (0, 0.3, 0.6, 1.2 mg/L) tested, the highest percentage of shoot buds induction (42.27%) and the highest number of induced shoot buds (6.42) per explant were observed when 0.6 mg/L TDZ was applied (Table 1 and Fig. 1A). Although the number of regenerated buds per explants was not small, most of the regenerated buds were very tiny and underdeveloped (Table 1 and Fig. 1A).

Table 1. Regeneration and elongation of adventitious buds from cotyledon explants of *J. curcas* with conventional culture methods.*

TDZ concentration (mg/L)* *	Regeneration of adventitious buds		Elongation of regeneration buds	
	Regeneration percentage (%)	Number of buds per explant	Mean shoot length (cm)	Number of shoot buds with more than 2 leaves per explant
0	0d	0e	0c	0c
0.1	19.28±2.19c	3.49±0.35d	0.72±0.14b	0.92±0.15ab
0.3	26.91±3.35b	4.71±0.56c	0.92±0.10ab	1.12±0.13a
0.6	42.27±4.12a	6.42±0.38a	1.12±0.16a	1.21±0.14a
1.2	31.55±2.61b	5.64±0.27b	0.62±0.15b	0.81±0.17b

Values represent means ± SD (standard deviation) of 25-30 explants per treatment in three independent experiments.

*To investigate the elongation effect of regenerated buds, mother tissues with regenerated shoot buds from conventional methods were transferred and inoculated onto fresh MS medium supplemented with 0.5 mg/L BA, 0.2 mg/L KT, 0.25 mg/L IAA and 0.4mg/L of GA3.

** Data in the same column followed by different letters are significantly different at $p \leq 5\%$ level as determined by Duncan's multiple range test.

Regeneration and Elongation of Adventitious Buds from Cotyledon Explants Treated with TDZ Solution before Culture

Explants Treated with 20 mg/L TDZ for Various Time Periods

To study the effect of time duration of TDZ treatment on adventitious buds induction, cotyledon explants treated with 20 mg/L TDZ for various time periods before inoculation of explants on the hormone-free MS medium. The results showed that time duration of the treatment significantly influenced the response of shoot buds induction (Table 2). Treatment with 20 mg/L TDZ solution for 40 min was the most suitable and it achieved the highest regeneration percentage (87.45%) and the largest number of regenerated buds per explant (11.23) (Table 2 and Fig. 1B). The percentage of induction of shoot buds and the number of induced shoot buds per explant were directly proportional to time duration of TDZ treatment when the time periods of TDZ treatment was not longer than 40 min. However, when the explants were treated with 20 mg/L TDZ solution for longer than 40 min, i.e. 60 min, the bud regeneration frequencies decreased significantly, then the percentage of shoot buds induction was 64.18%, and the number of induced shoot buds per explant was 9.02 (Table 2).

Table 2. Effect of treating explants with 20 mg/L TDZ solution for various time durations on the regeneration of adventitious buds from cotyledon explants of *J. curcas* after 30 days of culture.

Treating duration (min)	Regeneration percentage (%)	Number of buds per explant
0	0d*	0d
10	58.61±3.23c	6.25±0.36c
20	68.82±3.27b	8.42±0.38b
40	87.45±3.81a	11.23±0.49a
60	64.18±2.27b	9.02±0.43b
80	55.27±2.64c	6.98±0.15c

Values represent means ± SD (standard deviation) of 25-30 explants per treatment in three independent experiments.
* Data in the same column followed by different letters are significantly different at $p \leq 5\%$ level as determined by Duncan's multiple range test.

Regeneration and elongation of adventitious buds from cotyledon explants treated with various concentrations of TDZ solution before culture.

Cotyledon explants were treated with various concentrations of TDZ solution for 40 min before being inoculated onto hormone-free MS medium. The concentrations of TDZ solution significantly influenced the response of adventitious buds induction (Table 3 and Fig. 1B). The application of 20 mg/L TDZ resulted in the highest percentage of shoot bud induction (87.45%) and the highest number of induced shoot buds (11.23) per explant (Table 3 and Fig. 1B). The percentage of induction of shoot buds and the number of induced shoot buds per explant were directly proportional to the concentration of TDZ when the concentration of TDZ was not higher than 20 mg/L (Table 3). However, when TDZ was used at concentrations higher than 20 mg/L, the regeneration percentage of adventitious buds was decreased significantly (Table 3). Moreover, the elongation results of regenerated buds were better than the conventional methods (Table1 and Table 3), the best elongation of buds was from the cotyledon explants being inoculated onto MS medium after being treated explants with 20 mg/L TDZ solution for 40 min, and it achieved the greatest mean shoot length (1.83 cm) and the most number of shoot buds with more than 2 leaves per explant (3.23) (Table 3 and Fig. 1D).

Table 3. Effect of treating cotyledon explants with various concentrations of TDZ solution on the regeneration of adventitious buds in *J. curcas* after 30 days of culture.

	Regeneration of adventitious buds		Elongation of regeneration buds**	
TDZ solution concentration (mg/L)*	Regeneration percentage (%)	Number of buds per explant	Mean shoot length (cm)	Number of shoot buds with more than 2 leaves per explant
0	0e***	0e	0d	0d
10	56.28±3.12c	7.68±0.31c	1.56±0.14b	2.91±0.17a
20	87.45±3.81a	11.23±0.49a	1.83±0.12a	3.23±0.19a
30	69.42±3.54b	9.45±0.21b	1.66±0.13ab	3.02±0.27a
60	51.12±2.63c	7.74±0.34c	1.45±0.17bc	2.32±0.14b
120	25.47±2.71d	3.78±0.35d	1.19±0.15c	1.73±0.15c

Values represent means ± SD (standard deviation) of 25-30 explants per treatment in three independent experiments.
*All explants were treated with different concentrations of TDZ solution for 40 min.
** To investigate the elongation effect of regenerated buds, mother tissues with regenerated shoot buds from treatment with TDZ solution were transferred and inoculated onto fresh MS medium supplemented with 0.5 mg/L BA, 0.2 mg/L KT, 0.25 mg/L IAA and 0.4 mg/L of GA3.
***Data in the same column followed by different letters are significantly different at $p \leq 5\%$ level as determined by Duncan's multiple range test.

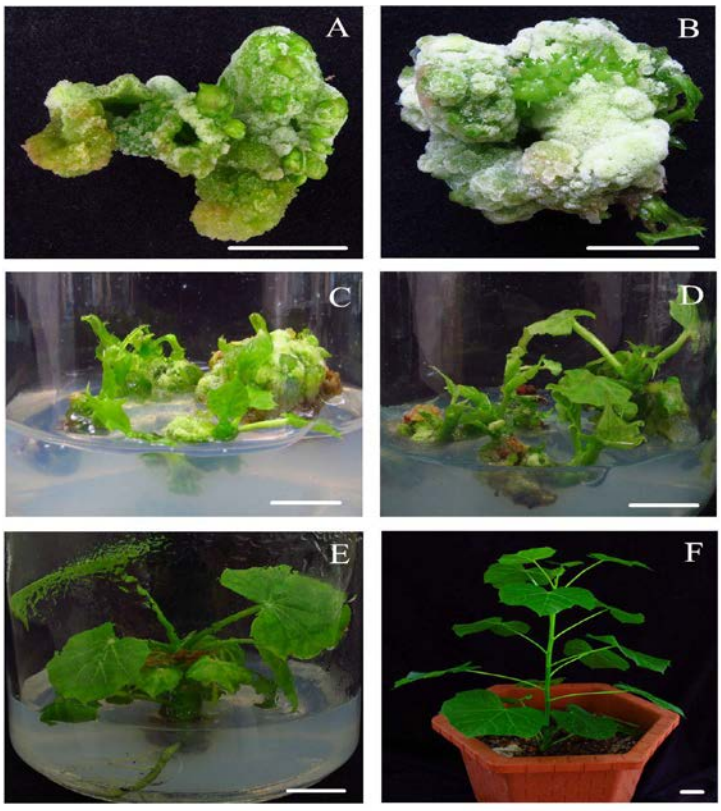

Figure. 1 Direct induction of shoot buds and elongation of regenerated buds from cotyledon explants of *J. curcas.* (A) Cotyledon explants were inoculated on MS medium containing 0.6 mg/L TDZ for 30 days (bar =0.5 cm); (B) Cotyledon explants were inoculated on hormone-free MS medium for 30 days after being treated with 20 mg/L TDZ solution for 40 min (bar =0.5 cm); Elongation of regenerated shoot-buds from conventional method (C) or treatment with TDZ solution (D) in MS medium supplemented with 0.5 mg/L 6-BA, 0.25 mg/L KT, 0.25 mg/L IAA and 0.4 mg/L GA3 after 15 days of culture (bar =1 cm); (E) Elongated shoot-buds were inoculated on half-strength MS medium supplemented with 0.1 mg/L IBA for 30 days (bar =1 cm); (D) A regenerated plant growing in a pot after acclimatization (bar =1 cm).

Influence of the IBA on rooting of the elongated shoot-buds

From the results summarized in table 4, it is clear that the influences of the IBA on the increase in rooting rate, root number and the average root length were all significant. What's more, when the rooting of shoots was being inoculated onto 1/2 MS medium supplemented with 0.1 mg/L IBA yielding the best results, and it achieved the highest of rooting rate (44.28%), the most number of roots (15.57) and the longest of average root length (6.24) after transplantation for 40 days of culture. This was better than the best results obtained by having been supplemented with other concentration of IBA in 1/2 MS medium (Table 4).

Table 4. Effects of various concentrations of IBA on rooting of the regenerated shoots from petiole explants of *J. curcas.*

Time (day)	IBA concentration (mg/l)	% rooting	No. root per shoot	Average root length (cm)
10	0	0c*	0c	0c
	0.1	2.32±2.35a	1.81±0.32a	1.43±0.09a
	0.3	1.68±2.18b	0.68±0.56b	0.51±0.48b
	0.6	0c	0c	0c
	1.2	0c	0c	0c
15	0	0d	0d	0d
	0.1	21.87±2.86a	3.87±0.59a	2.35±0.21a
	0.3	18.32±1.74ab	1.29±0.32b	0.99±0.12b
	0.6	5.47±4.75c	0.72±0.53bc	0.68±0.46bc
	1.2	3.86±3.25c	0.47±0.31c	0.45±0.22c
20	0	0e	0e	0d
	0.1	34.64±3.56a	5.42±0.58a	3.54±0.47a
	0.3	26.74±2.58b	4.52±0.14ab	2.36±0.24b
	0.6	15.65±3.61cd	1.22±0.24cd	0.87±0.09c
	1.2	8.65±3.57d	0.78±0.24d	0.61±0.11c
30	0	0e	0e	0d
	0.1	42.67±1.54a	8.38±0.92a	4.27±0.31a
	0.3	29.54±2.25b	6.55±1.39b	3.68±0.73a
	0.6	21.44±3.25c	1.72±0.43cd	2.45±0.23b
	1.2	14.42±3.31d	1.05±0.43d	1.42±0.24c
40	0	0e	0d	0e
	0.1	44.28±0.87a	15.57±0.86a	6.32±0.74a
	0.3	31.26±2.42b	8.58±1.13b	3.85±0.23b
	0.6	23.25±2.03c	3.27±0.73c	2.81±0.12c
	1.2	16.25±2.74d	2.14±0.58c	1.69±0.27d

Values represent means ± SD (standard deviation) of 30 explants per treatment in three independent experiments.
*Data in the same column of the same culture time followed by different letters are significantly different by Duncan's test at p ≤5% level.

Discussion

Genetic transformation helps to modify one or a few traits of the species at a time. Whilst tissue culture methods for inducing plant regeneration from cotyledon explants are very important for transformation. Conventional culture methods for inducing adventitious buds regeneration from *J. curcas* explants included direct inoculation of the explants on a medium containing cytokinin such as TDZ at low concentrations (usually 0.3-1.2 mg/L) [20-22]. These methods showed low regeneration efficiency and the regenerated buds were hardly to further elongation and growth (Table 1 and Figs.

1A and C). However, treatment of cotyledon explants with TDZ solution at high concentrations (10-60 mg/L) in this study for a short time periods (10-60 min) before inoculation on hormone-free MS medium increased the regeneration frequency and caused the formation of bigger buds as compared with the conventional methods, further culture of the regenerated buds showed that the regenerated buds were easily elongated (Table 3 and Figs. 1B and D).

TDZ belongs to the cytokinin family of hormones. In plant tissue culture, cytokinin is an essential factor for the induction of adventitious buds formation in most cases [20-22,25]. Our study suggested that cytokinin might be required for a short duration during induction of shoot-buds formation after high concentration of TDZ treatment.The success of experimental results given in this paper suggested that the induction of adventitious buds formation might not requires cytokinin during the whole culture period; when the process of cell division for the formation of adventitious buds was triggered, the presence of cytokinin was no longer necessary, and in the contrary, long presence as incorporated in the medium might have only negative effects. What's more, it has been well documented in many text books and academic journals that cytokinin has the effects of apical suppression and hinders the growth of roots [26,27].

In our previous study, high concentrations (30 mg/L) of BA treatment for 20 min before inoculating the explants on hormone-free MS medium increased the buds regeneration frequency from hypocotyl explants in soybean [26]. These results suggested that the new culture method might be applicable to a wide range of plant speies and different types of explant, although different cytokinins should be tested for better results.

In conclusion, an efficient plant regeneration protocol was established for *J. curcas* using cotyledon explants (Figs. 1A-F). These methods have the potential to facilitate the genetic modification and subsequent *in vitro* multiplication of *J. curcas* cultivars for various uses, Furthermore, the technology can lead to a better understanding and improvement of the biofuel species, which have positive implications on reducing the world's dependence on fossil reserves.

Summary

An efficient and reproducible protocol was developed of *J. curcas* using cotyledon explants. The best shoot buds induction was seen in the cotyledon explants treated with 20 mg/L TDZ solution for 40 min, followed by 30 day culture on hormone-free MS medium. An intact plantlet could usually be obtained in 60 days of culture by using the culture protocol described in this study.

Acknowledgements

This work were supported by the National Science Foundation of P. R. China (31271759), the Project of Science and Technology of Guangdong province (2013B060400024 and 2014A020208116), the Program for Scientific Research Start-up Funds of Guangdong Ocean University, and the Project of Science and Technology of Zhangjiang city (2016B101).

References

[1] A.S. Helmy Attaya, D. Geelen, H. Belal and A. El-Fatah, Progress in *Jatropha curcas* tissue culture, Am. -Eurasian J. Sustain. Agric. 6 (2012) 6-13.

[2] B.Schmook and L. Serralta-Peraza, Jatropha curcas: distribution and uses in the Yucatan Peninsula of Mexico, In Gubitz, G. M., Mittelbach, M., and Trabi, M., (ed.), Biofuels and industrial products from *Jatropha curcas*, Dbv-Verlag, Graz, 1997, pp. 53-57.

[3] P.H. Joubert, J.M. Brown, I.T. Hay and P.D. Sebata, Acute poisoning with *Jatropha curcas* (purging nut tree) in children, S. Afr. Med. J. 65 (1984)729-730.

[4] H.P.S. Makkar, A.O. Aderibigbe and K. Becker, Comparative evaluation of non-toxic and toxic varieties of *Jatropha curcas* for chemical composition, digestibility, protein degradability and toxic factors, Food Chem. 62 (1998) 207-215.

[5] R.G. Menezes, N.G. Rao, S.S. Karanth, A. Kamath, S. Manipady and V.V. Pillay, *Jatropha curcas* poisoning, Ind. J. Pediatr. 73 (2006) 634.

[6] M.M. Datta, P. Mukherjee, B. Ghosh and T.B. Jha, In vitro clonal propagation of biodiesel plant *(Jatropha curcas* L.), Curr. Sci. 93 (2007) 1438-1442.

[7] R.C. Gupta, Pharmacognostic studies on 'Dravanti'. Part I. *Jatropha curcas* Linn, Proc. Plant Sci. 94 (1985) 65-82.

[8] K. Openshaw, A review of *Jatropha curcas*: an oil plant unfulfilled promise, Biomass Bioenergy. 19 (2000) 1-15.

[9] K.O. Adebowale and C.O. Adedire, Chemical composition and insecticidal properties of the underutilized *Jatropha curcas* seed oil, Afr. J. Biotechnol. 10 (2006) 901-906.

[10] A.A.A. Liberalino, E.A. Bambirra, T. Moraes-Santos and E. C. Vieira, Jatropha curcas L. seeds: chemical analysis and toxicity, Arq. Biol. Technol. 31 (1998) 539-550.

[11] F.K. Forson, E.K. Oduro and E. Hammond-Donkoh, Performance of *Jatropha* oil blends in a diesel engine, Renew Energy. 29 (2004) 1135-1145.

[12] R. Banerji, A.R. Chowdhury, G. Misra, G. Sudarsanam, S.C. Verma and G.S. Srivastava, *Jatropha* seed oils for energy, Biomass. 8 (1985) 277-282.

[13] A. Ghosh, D.R. Chaudhary, M.P. Reddy, S.N. Rao, J. Chikara, J.B. Pandya, J.S. Patolia, M.R. Gandhi, S. Adimurthy, N. Vaghela, S. Mishra, M.R. Rathod, A.R. Prakash, B.D. Shethia, S.C. Upadhyay, V. Balakrishna, C.R. Prakash and P.K. Ghosh, Prospects for *Jatropha* methyl ester (biodiesel) in India, Int. J. Environ Stud. 64 (2007) 659-674.

[14] A. Singh, M.P. Reddy, J. Chikara and S. Singh, A simple regeneration protocol from stem explants of *Jatropha curcas*-a biodiesel plant, Ind. Crops Prod. 31 (2010) 209-213.

[15] B. Jaganath, K. Subramanyam, S. Mayavan, S. Karthik, D. Elayaraja, R. Udayakumar, M. Manickavasagam and A. Ganapathi, An efficient in planta transformation of Jatropha curcas (L.) and multiplication of transformed plants through *in vivo* grafting, Protoplasma. DOI 10.1007/s00709-013-0558-z (2013).

[16] M. Sujatha and N. Mukta, Morphogenesis and plant regeneration from tissue cultures of *Jatropha curcas*, Plant Cell Tiss. Organ Cult. 44(1996) 135-141.

[17] J. Lin, L. Tang and F. Chen, Tissue culture and plantlet regeneration of *Jatropha curcas*, Plant Physiol. Commun. 38 (2002) 252-256.

[18] W.D. Lu, Q.Wei, L. Tang, F. Yan and F. Chen, Induction of callus from *Jatropha curcas* and rapid propagation, Chin. J. Appl. Environ. Biol. 9 (2003) 127-130.

[19] Q. Wei, W.D. Lu, Y. Liao, S.L. Pan, Y. Xu, L. Tang and F. Chen, Plant regeneration from epicotyl explants of *Jatropha curcas*, J. Plant Physiol. Mol. Biol. 30 (2004) 475-478.

[20] N. Khemkladngoen, J. Cartagena, N. Shibagaki and K. Fukui, Adventitious shoot regeneration from juvenile cotyledons of a biodiesel producing plant *Jatropha curcas* L., J. Biosci. Bioeng. 111 (2011) 67-70.

[21] N. Kumar and M.P. Reddy, Thidiazuron (TDZ) induced plant regeneration from cotyledonary petiole explants of elite genotypes of *Jatropha curcas*: A candidate biodiesel plant, Ind. Crops. Prod. 39 (2012) 62-68.

[22] N. Kumar, K.V. Anand and M.P. Reddy, Shoot regeneration from cotyledonary leaf explants of Jatropha curcas: a biodiesel plant, Acta. Physiol. Plant. 32 (2010) 917-924.

[23] A.C. Deore and T.S. Johnson, High-frequency plant regeneration from leaf-disc cultures of *Jatropha curcas* L.: an important biodiesel plant, Plant Biotech. Rep. 2 (2008) 10-15.

[24] T. Murashige and F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant. 15 (1962) 473-479.

[25] V. Khurana-Kaul, S. Kachhwaha and S.L. Kothari, Direct shoot regeneration from leaf explants of *Jatropha curcas* in response to thidiazuron and high copper contents in the medium, Biol. Plant. 54 (2010) 369-372.

[26] Y. Liu, L. Yu, Q. Zhang, H. Nian, Z.F. Guo and Y.S. Yang, High concentration short duration treatment of benzyladenine stimulates adventitious bud regeneration from hypocotyl explants in soybean, Adv. Mater. Res. 647 (2012) 331-335.

[27] Y. Liu, X. Tong, W. Hui, T. Liu, X. Chen, J. Li, C.X. Zhuang, Y.S. Yang and Z.L. Liu, Efficient culture protocol for plant regeneration from petiole explants of physiologically mature trees of *Jatropha curcas* L, Biotechnol. Biotec. Eq. 29 (2015) 479-488.

