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AN EFFICIENT PROTOCOL FOR INDUCING REGENERATION IN PHYSIC NUT (*JATROPHA CURCAS* L.)

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Abstract

An efficient protocol for induction of adventitious buds from petiole explants of *Jatropha curcas* was developed. Direct culturing of petiole explants from young seedlings on medium with thidiazuron (TDZ) from 0.1 to 1.2 mg/l induced only poor quality adventitious buds with low regeneration frequencies. However, by treating the petiole explants with 20 mg/l TDZ solution for 20 min before inoculating the explants to the medium, the regeneration rate was increased to as high as 91.36% as well as 12.01 shoot-buds per explant. Furthermore, by *in vitro* grafting the elongated shoots to seedling stocks and culturing the grafted shoots on half-strength MS medium containing 0.3 mg/l IBA and 2 mg/l glutamine. Intact plantlets ready for transplantation to the soil was obtained in 20 days eventually.

Introduction

Physic nut (*Jatropha curcas* L.) is a woody plant belongs to Euphorbiaceae widely distributed in the sub-tropical and tropical regions (Helmy Attaya *et al*. 2012, Schmook and Serralta-Peraza 1997). This tree is world-renowned for the high content (40 to 60%) of excellent oil in its seeds (Liberalino *et al*. 1998). Numerous reports showed that the methyl ester in seed oil could be processed to produce biodiesel to substitute the conventional fossil diesel easily, and for this reason, there has been enormous interest in cultivation of *J. curcas* around the world (Banerji *et al*. 1985, Forson *et al*. 2004, Ghosh *et al*. 2007).

Physic nut is usually propagated by seeds. Development of conventional breeding has been impeded with low and inconstant seed production by reasons of heterozygous of *J. curcas* plants and sexual incompatibilities due to pernicious gene linkages (Singh *et al*. 2010, Jaganath *et al*. 2013). Seed germination rate is very poor due to many reasons. This conventional method is not enough for large scale propagation.

Previously, regeneration of shoot-bud has been claimed by the application of conventional protocol of inoculating explants on media supplemented with cytokinin (Sujatha and Mukta 1996, Lin *et al*. 2002, Lu *et al*. 2003, Wei *et al*. 2004, Deore and Johnson 2008, Kumar *et al*. 2010, Khemkladngoen *et al*. 2011, Kumar and Reddy 2012). However, we found it very difficult to repeat the results with our *J. curcas* genotypes. With all these considerations present work was carried out to develop an efficient *in vitro* propagation protocol for *J. curcas* through petiole explant culture.

Materials and Methods

Mature seeds of a *J. curcas* clone named M-19 were collected from the farm in Haikou, Hainan province of China (Liu *et al*. 2015). The seeds were then sowed in moist soil, and petiole explants were prepared from 20-day-old seedlings which had been surface-sterilized with 2%

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sodium hypochlorite (NaClO) for 20 min and rinsed five times in sterile distilled water. Media used in the present experiments contained 2.5% sucrose that were adjusted to pH 5.8 - 6.0 and had 0.7% agar added prior to autoclave at 1.4 kg/cm^2 for 20 min. All culture treatments were kept at 25 ± 1ºC under a 12 hrs photoperiod of 60 - 80 µmol m^2/s intensity (cool white fluorescent tubes).

TDZ was dissolved in 1 mol/l NaOH solution in advance, and then adjusted with 1 mol/l NaOH or 1 mol/l HCl solution to have a pH value range of 5.8 - 6.0. This TDZ stock solution was further diluted with distilled water to yield 0, 5, 10, 20, 30, 60 and 120 mg/l. These TDZ solutions were then filter-sterilized and kept in a refrigerator before use. Petiole explants were soaked in glass bottles filled with TDZ solutions of different concentrations for various durations (0, 5, 10, 20, 40 and 80 min). After the treatment, the explants were momentarily placed on sterile dry filter paper to remove water on the surface. In order to induce regeneration of shoot-buds, petiole explants were inoculated onto hormone-free MS medium after being treated with TDZ solution for different time periods. For comparing, petiole explants were treated by conventional methods of directly inoculating the explants onto MS medium supplemented with various concentrations of TDZ (0, 0.1, 0.3, 0.6 and 1.2 mg/l) (Kumar *et al*. 2010, Khemkladngoen *et al*. 2011, Kumar and Reddy 2012). 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 GA_3, 0.5 mg/l BA, 0.2 mg/l Kn and 0.25 mg/l IAA for 15 days (Deore and Johnson 2008, Liu *et al*. 2015).

Regenerated shoots (0.5 cm or longer) were isolated from the mother tissues as scions and hypocotyls with 0.2 cm radical prepared from 5 days old *J. curcas* seedlings after surface-sterilization were served as stocks for the grafting. The upper surface of the hypocotyls stocks was cut across at the middle to about 5 mm depth, and then one shoot scion was cut to have a "V" shape base and inserted into the cutting. Grafted seedlings were inoculated onto fresh half-strength MS medium supplemented with 0, 0.3 and 0.6 mg/l of IBA and 0, 2 and 6 mg/l glutamine for 20 days.

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 by using SPSS 17.0 soft ware, and data were significantly different at $p \leq 5\%$ level as determined by Duncan's multiple range test. The results were displayed by means ± Sd of three separate experiments.

Results and Discussion

Petiole explants were inoculated onto MS medium supplemented with different concentrations of TDZ as reported previously (Kumar *et al*. 2010, Khemkladngoen *et al*. 2011, Kumar and Reddy 2012). The highest percentage (63.68) of shoot-bud regeneration and the largest number (6.58) of regenerated buds per explant were surveyed when 0.3 mg/l TDZ was applied (Table 1 and Fig. 1A and B). Though the number of regenerated buds per explants was not few, the majority of adventitious buds were very small and hard to elongate (Table 1 and Fig. 1C).

Explants treated with 20 mg/l TDZ for various durations. For the purpose of studying the influence of time duration of TDZ solution treatment on the induction of adventitious buds, petiole explants were treated with 20 mg/l TDZ for different durations before inoculation of explants onto the hormone-free MS medium. Results indicated that treatment with 20 mg/l TDZ solution for 20 min was the most suitable, by which the highest regeneration percentage (91.36) and the largest number of regenerated buds per explant (12.01) were obtained (Table 1 and Fig. 1D and E). Nevertheless, when the explants were treated with 20 mg/l TDZ solution for longer than 20 min, i.e. 40 min, the regeneration frequencies of adventitious buds were reduced (Table 2).

Regeneration and elongation of adventitious buds from petiole explants treated with different concentrations of TDZ solution before culture. Petiole explants were treated with different concentrations of TDZ solution for 20 min. Results of the experiment (Table 3) indicated that the highest percentage (91.36) of shoot-bud induction and the largest number (12.01) of induced shoot-bud per explant were obtained when explants were treated with 20 mg/l TDZ solution (Table 3 and Fig. 1D and E). In contrast, when TDZ was applied at concentrations higher than 20 mg/l, the induction rate of shoot-buds was reduced dramatically. In addition, by the new culture methods, the elongation of shoot-bud was improved largely (Table 3 in comparison with Table 1). The best elongation result was obtained by treating explants with 20 mg/l TDZ solution for 20 min, which obtained the best mean shoot length (1.79 cm) and the largest number (4.21) of high quality shoot-bud (with more than 2 leaves per explant) (Table 3 and Fig. 1F).

Table 1. Regeneration and elongation of adventitious buds with conventional culture methods.*

TDZ conc. (mg/l)**	Regeneration of adventitious buds		Elongation of regeneration buds	
	Regeneration (%)	No. of buds/explant	Mean shoot leng (cm)	Number of shoot buds with more than 2 leaves/explant
0	0d***	0d	0c	0c
0.1	52.72 ± 2.13b	5.24 ± 0.48b	0.74 ± 0.14b	0.92 ± 0.16ab
0.3	63.68 ± 3.37a	6.58 ± 0.66a	1.12 ± 0.16a	1.20 ± 0.12a
0.6	60.07 ± 4.45a	6.12 ± 0.54a	0.90 ± 0.11ab	1.11 ± 0.15a
1.2	41.34 ± 3.15c	4.23 ± 0.52c	0.62 ± 0.15b	0.81 ± 0.17b

*Values represent means ± Sd of 25 - 30 explants per treatment in three independent experiments. **To investigate the elongation effect of adventitious buds, mother tissues with regenerated shoot-bud from conventional methods were transferred and inoculated on fresh MS medium containing 0.5 mg/l BA, 0.2 mg/l KT, 0.25 mg/l IAA and 0.4 mg/l of GA_3. ***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.

Table 2. Results of treating explants with 20 mg/l TDZ solution for various period on the regeneration of adventitious buds.*

Treating duration (min)	Regeneration (%)	Number of buds per explant
0	0d**	0d
5	57.12 ± 2.63c	6.55 ± 0.33c
10	73.12 ± 4.27b	7.58 ± 0.51b
20	91.36 ± 3.66a	12.01 ± 0.46a
40	74.44 ± 4.24b	8.01 ± 0.57b
80	55.47 ± 3.19c	6.05 ± 0.28c

*Values represent means ± Sd 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.

For grafting the regenerated shoots to obtain intact plants faster, 0.5 cm or longer shoots were isolated as scions and hypocotyls with about 0.2 cm radical were prepared from 5 days old surface-sterilized seedlings of *J. curcas* as stocks. The grated seedlings which were inoculated onto

half-strength MS medium containing 0.3 mg/l IBA and 2 mg/l Gln yielded the best growth results; the highest grafted seedlings (1.87 cm), the largest number of roots (8.15), and the highest survival rate (78.37%) after transplantation (Table 4, Fig. 1G). This result was significantly better than on half-strength MS medium supplemented with only IBA or Gln. On the other hand, when the grafted seedlings were inoculated on 1/2 MS medium without IBA and without Gln supplementations, the results of growth were very poor (Table 4). By application of the grafting method and proper culture of the grafted seedlings, healthy intact plantlets could be reliably recovered after a simple acclimatization step (Fig. 1H and I).

Table 3. Results of treating explants with different concentrations of TDZ solution on the regeneration of adventitious buds.*

TDZ conc. (mg/l)**	Regeneration of adventitious buds		Elongation of regeneration buds***	
	Regeneration (%)	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
5	64.77 ± 4.06c	7.25 ± 0.42c	1.42 ± 0.12b	2.38 ± 0.39bc
10	77.78 ± 3.09b	8.46 ± 0.35b	1.56 ± 0.13ab	3.05 ± 0.18b
20	91.36 ± 3.66a	12.01 ± 0.46a	1.79 ± 0.12a	4.21 ± 0.23a
30	79.07 ± 4.65b	9.19 ± 0.52b	1.63 ± 0.11ab	3.25 ± 0.24b
60	63.33 ± 3.67c	6.18 ± 0.83c	1.46 ± 0.17bc	2.37 ± 0.45bc
120	24.49 ± 2.46	3.04 ± 0.38d	1.25 ± 0.10c	1.98 ± 0.17c

*Values represent means ± Sd of 25 - 30 explants per treatment in three independent experiments. **All explants were treated with different concentrations of TDZ solution for 40 min. ***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 GA_3. ****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.

Plant genetic transformation contributes to alter some traits in species. Meanwhile, tissue culture protocol for inducing regeneration of adventitious buds from explants is in most of the cases a prerequisite for successful transformation. Conventional methods for inducing regeneration of adventitious buds from petiole explants in *J. curcas* directly inoculated explants of a few kinds onto media supplemented with TDZ at low concentrations (Kumar *et al*. 2010, Khemkladngoen *et al*. 2011, Kumar and Reddy 2012). Those results prove that the regeneration of adventitious buds was of low-efficiency and further elongation and growth of the regenerated buds were considerable difficult. Nevertheless, when petiole explants were treated with 20 mg/l TDZ solution for 20 min before inoculation onto hormone-free MS medium as in the present experiments, the regeneration frequency was evidently raised and buds of much better quality were induced.

In present study, the results showed that TDZ was needed for only a short period for the induction of adventitious buds, implicating that when the procedure of cell division was activated for the formation of adventitious buds, the existence of TDZ might be no longer indispensable and do harm to the generation and development of adventitious buds. In addition, it has been recorded that extra TDZ in medium might have the effectiveness of suppression of the growth of roots (Liu *et al*. 2012, Liu *et al*. 2015).

In our previous study, by treating hypocotyl explants with 30 mg/l BA solution for 20 min before inoculating the explants onto hormone-free MS medium, the regeneration frequency of buds was raised observably in soybean [*Glycine max* (Linn.) Merr.] (Liu *et al*. 2012). These results implied that the new culture protocol could be applicable for different plant species and extensive kinds of explants. It has been documented in a broad range of plant species in *in vitro* cultures that elongation of regenerated buds was not easy to achieve (Sujatha and Mukta 1996, Lin *et al*. 2002,

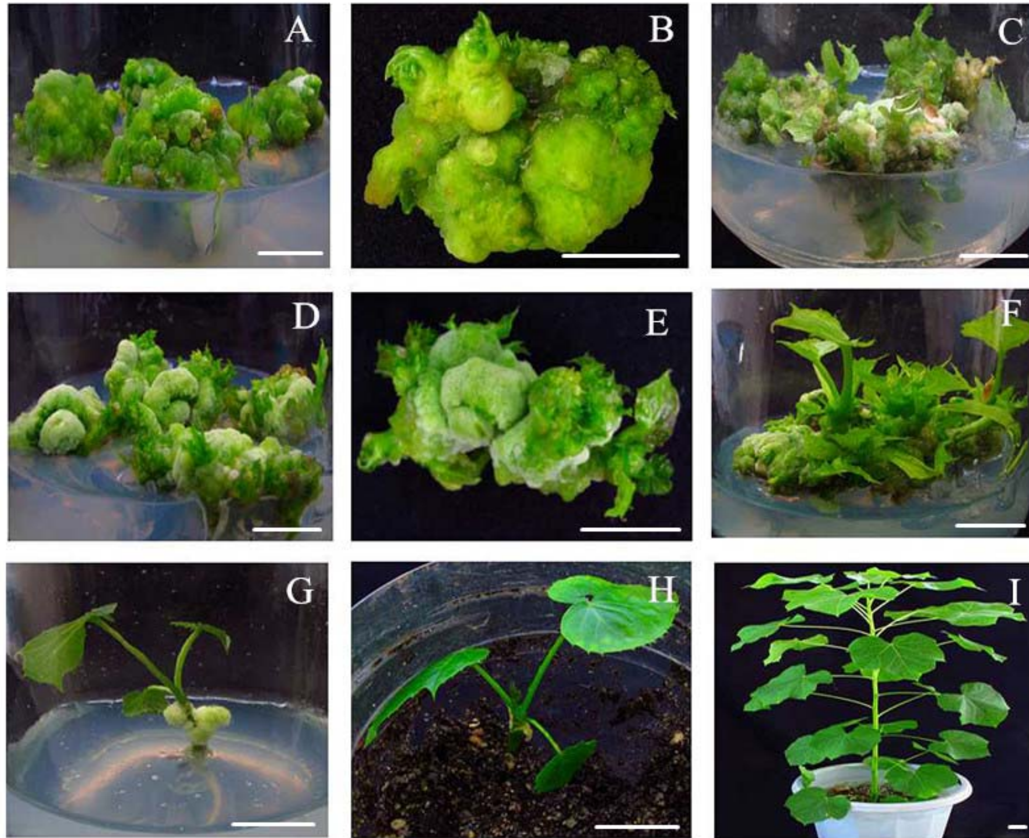

Fig. 1. Induction of adventitious buds and elongation of regenerated buds from petiole explants, and the growth of an *in vitro* grafted plantlet in *J. curcas.* Petiole explants (A) and (B) were inoculated on MS medium containing 0.3 mg/l TDZ for 30 days; Petiole explants (D) and (E) were inoculated onto hormone-free MS medium for 30 days after being treated with 20 mg/l TDZ solution for 20 min; Elongation of regenerated shoot-buds by conventional method (C) or treatment with TDZ solution (F) 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 GA_3 after 15 days of culture; Growth of a grafted plantlet (G) in half-strength MS medium supplemented with 0.3 mg/l IBA and 2 mg/l Gln at 20 days; A grafted plantlet in a plastic bottle 10 days after transplantation (H) and in a pot 50 days after transplantation (I). (Bar =1 cm)

Lu *et al*. 2003, Wei *et al*. 2004, Deore and Johnson 2008, Kumar *et al*. 2010, Khemkladngoen *et al*. 2011, Kumar and Reddy 2012), however, by grafting the elongated buds to suitable stocks, the growth of the grafted buds could be effectively enhanced, and then entire plantlets could be gained in a short period of time.

Table 4. Results of influence of the IBA and glutamine on the growth of grafted seedlings.*

IBA conc. (mg/l)	Glutamine (mg/l)	Survival rate of grafting (%)	Increase in height (cm)	No. of root increased	Survival rate after transplantation (%)
0	0	86.65 ± 4.51b**	0.91 ± 0.06d	1.76 ± 0.09d	54.75 ± 3.14c
0.3	0	100a	1.55 ± 0.07b	5.84 ± 0.30b	70.56 ± 2.53ab
0.6	0	100a	1.34 ± 0.12c	4.89 ± 0.14b	68.71± 3.14b
0	2	90.27 ± 3.56b	1.25 ± 0.11c	2.85 ± 0.23c	58.63 ± 3.72c
0	6	88.36 ± 4.63b	1.17 ± 0.08c	1.94 ± 0.16d	56.61 ± 2.73c
0.3	2	100a	1.87 ± 0.09a	8.15 ± 0.57a	78.37 ± 3.26a
0.3	6	100a	1.75 ± 0.07a	5.71 ± 0.53b	70.25 ± 3.54ab

*Values represent means ± Sd 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.

A high-efficient plant regeneration method was developed by using petiole explants in *J. curcas*. The highest induction efficiency of adventitious buds was observed when petiole explants were treated with 20 mg/l TDZ solution for 20 min before inoculation on hormone-free MS medium. In order to recover the intact plantlets, regenerated shoots could be grafted and cultured on half-strength MS medium supplemented with 0.3 mg/l IBA and 2 mg/l Glutamine. By this way, an intact plantlet could be obtained in a short culture period of 65 days. This newly established protocol was highly efficient for cloning *J. curcas* plantlets and might be applicable for *J. curcas* genetic transformation studies.

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