

Efficient Protocol for Introducing Regeneration of Adventitious Buds from Hypocotyl Explants in *Jatropha curcas* L.

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Abstract. A new strategy of high concentration short duration treatment of Thidiazuron (TDZ) for enhancing adventitious buds formation from hypocotyl explants were attempted in *Jatropha curcas*. The best shoot buds induction (81.91%) and number of buds per explant (10.16) were obtained from hypocotyl explants treated with 20 mg/L TDZ solution for 20 min, followed by 30 days of culture on hormone-free MS medium. The elongated shoots initiated roots to become intact plantlets in rooting medium containing indole-3-butyric acid (IBA) and L-glutamine (Gln), and the results showed that the initiation and growth of roots were effectively stimulated with supplementing 16 mg/L Gln into the rooting medium, and having the best rooting rate (51.39%). After acclimatization, an intact plantlet could usually be gained at 60 days of culture by using the culture protocol described in this study.

Introduction

Jatropha curcas L. is a sort of woody plant and pertains to the *Euphorbiaceae* family [1,2]. As is well-known that the latex from *J. curcas* contains alkaloids have been reported to contain medical composition and can be utilized to acquire pharmaceutical adjective constituents [3,4] and pesticides [5]. Nevertheless, the best known of this tree is the high oil yielding from its seeds (40% to 60%) [6]. Widespread interest in cultivation of *J. curcas* all over the world, because methyl ester extracted from *J. curcas* is suitable for production of biodiesel [4,5,7,8].

The most major problem for *J. curcas* plants not being grown on a major scale is that the low and unstable seed production because of natural hybrid [9]. With the purpose of breeding excellent varieties, the method of genetic transformation of the species might be a feasible approach [10-13].

In a general way, adventitious shoot regeneration from the transformed receptors (explants) is a precondition in genetic transformation system. Moreover, it is reported that the tissue culture cycles of common regeneration protocols were quite time-consuming (usually 3-5 months) for gaining intact plant in most of the reports in *J. curcas*. Consequently, it is indispensable to exploit high-efficiency methods of tissue culture, which could accelerate mass production of good-quality implants materials and capacitate breed improvement by means of technique for gene engineering.

It was found that the regeneration of plants from different organs as explant sources had been implemented using tissue cultures protocols in *J. curcas* [14-19]. However, the regeneration efficiency of hypocotyl explants was barely satisfactory in *J. curcas* [20-23].

Materials and Methods

Plant Materials

The mature *J. curcas* seed coded M-19 were the material source in our study. All plants were cultivated at a farm in Haikou, China [24].

Preparation of Explants

Vigorously growing *in vitro* hypocotyl from 5 day old aseptic seedlings were cut into *in vitro* hypocotyl explants of about 0.5 cm in length. Well-growing hypocotyl from 12 day old seedlings were cut into *in vivo* hypocotyl explants of about 0.5 in length.

Preparation of Thidiazuron (TDZ) Solution

TDZ was dissolved into 1 mol/L NaOH to produce 0.2 mg/mL TDZ solution. And the solution was diluted with distilled water to generate the concentrations as follows: 0, 10, 20, 30 and 60 mg/L. These were adjusted with 1 mol/L HCl to acquire a pH value range of 5.8-6.0, and filter-sterilized before being used for treating the hypocotyl explants.

Dealing with the Hypocotyl Explants with TDZ Solution

Hypocotyl explants were immersed in glass petri dishes supplemented with various concentrations (0, 10, 20, 30 and 60 mg/L) of TDZ solution for various time periods (0, 5, 20, 30 and 40 min). After treatment, the explants were transitorily put on sterile absorbent paper to soak excess amount of moisture.

Preparation of Mediums and Culture Conditions

Ordinary MS media was applied to overall tissue culture experiments in this study. All mediums had been included 2.5 % sucrose and 0.6% agar, and tuned to pH 5.8-6.0 with 1 mol/L NaOH before being autoclaved at 1.4 kg cm⁻² for 20 minutes. Unified culture conditions were applied in all experiments. All treatments were kept at 25 ± 1°C under a 12 h photoperiod of 60-80 μ mol m⁻²s⁻¹ intensity (cool white fluorescent tubes).

Regeneration of Adventitious Buds

In order to induce adventitious buds regeneration, hypocotyl explants were placed onto hormone-free Murashige and Skoog's (MS) medium [25] after treated by TDZ solution for different time periods for 30 days. The explants were put on the mediums with their morphological up-side up vertically simply for comparative experiments. What's more, hypocotyl explants were also treated with conventional approach and horizontally inoculated onto MS medium including 0, 0.1, 0.3, 0.6 and 1.2 mg/L TDZ as reported anteriority [20-23].

Elongation of Shoot-Buds

The regenerated shoot-buds were transferred with the mother tissues (explants) together onto MS medium containing 0.5 mg/L BA, 0.25 mg/L indole-3-acetic acid (IAA), 0.2 mg/L kinetin (KT) and 0.4 mg/L gibberellic acid (GA₃) for 15 days [24,26].

Rooting

For the sake of inducing rooting of the elongated shoot-buds, shoots (at least 10 mm in length) were separated from the maternal tissues and inoculated them on MS medium contained 0.3 mg/L indole-3-butyric acid (IBA) and various concentrations (0, 2, 8, 16, 24 and 48 mg/L) of L-glutamine (Gln) and the experimental results were recorded after cultivating 10, 20, 30 and 40 days. Gln solution was filter-sterilized and supplemented into the autoclaved medium as depicted anteriorly [27].

Process of Transplant Acclimatization

Regenerated plantlets were taken out from jars carefully, washed completely for wiping off

residuary mediums, and then they were migration to plastic pots with germfree sand and soil at the ratio 1:1 and carpeted with plastic wrap for 15-20 days. Finally, the complete plants were moved to a conservatory (working Humidity 70-80% and temperature $25 \pm 3^{\circ}\text{C}$) for further accumulation and growth.

Data Analysis

All experiments were established on a design of random factor method completely and repeated 3 times with 35 explants per treatment in the present study. 'Regeneration percentage' was computed by dividing the number of explants that regenerated shoot-buds with the amount of explants. 'Number of buds per explant' was computed by dividing the number of regenerated shoot-buds with the amount of hypocotyl explants that had regenerated buds. 'Length of shoot-buds' was computed by the average length of elongated shoots. 'Rooting percentage' was computed by dividing the number of shoots that had regenerated adventitious roots with the total amount of shoots used for inducing rooting. 'Number of roots per shoot' was computed by dividing the number of regenerated roots with the amount of shoots that had regenerated roots. 'Average root length' was computed by dividing length of all regenerated roots with the number of regenerated roots. Statistical analysis of the data was implemented by utilizing SPSS 17.0 soft ware. Furthermore, data in the same column followed by different letters was significantly different by Duncan's test at $p \leq 5\%$ level. The experimental data was shown as means \pm SD (standard deviation) of 3 standalone experiments.

Results

Regeneration of Shoot-Buds from Hypocotyl Explants with Conventional Techniques

Hypocotyl explants were inoculated on MS medium supplemented with different concentrations of TDZ as reported anteriorly [20-23]. The results indicated that the highest centage of adventitious buds induction (24.73%) and the maximum number of induced shoot-buds per explant (2.63) were investigated when 0.6 mg/L TDZ was used (Table 1 and Fig. 1A). However, improving the TDZ concentration to exceed 0.6 mg/L would not obtain better results, so 0.6 mg/L TDZ was the most suitable concentration for the induction of shoot-buds (Table 1 and Fig. 1A). Furthermore, the quantity of regenerated buds per explants was considerably small, whilst majority of the regenerated shoot-buds were extremely tiny and underdeveloped. What's more, further elongation of shoot-buds was very difficult at 0.6 mg/L concentration (data not shown).

Table 1. Results of various TDZ concentrations on the regeneration of adventitious buds from hypocotyl explants in *J. curcas* after 30 days of culture.

TDZ concentration (mg/L)	Regeneration percentage (%)	Number of buds per explant
0	0d	0d
0.1	9.93 \pm 1.81c	1.19 \pm 0.12c
0.3	18.80 \pm 1.93b	1.81 \pm 0.32b
0.6	24.73 \pm 3.06a	2.63 \pm 0.21a
1.2	17.97 \pm 2.38b	2.13 \pm 0.28ab

Regeneration of Shoot-Buds from Hypocotyl Explants after Being Treated with TDZ Solution

Hypocotyl explants were dealt with different concentrations of TDZ solution for 20 min before being placed onto basic MS medium free of hormone. The experimental results showed clearly that the protocol of treatment with TDZ solution was more efficient than conventional means on induction of shoot-buds regeneration from hypocotyl explants. Moreover, the highest percentage of adventitious buds induction (81.91%) and the maximum number of induced shoot-buds per explant (10.16) were obtained on account of the utilization of 20 mg/L TDZ solution (Table 2 and Fig. 1B).

When the concentrations of TDZ solution were not more than 20 mg/L, the percentage of adventitious buds induction and the number of induced shoot-buds per explant were proportional to the TDZ concentrations directly. Nevertheless, the application of higher than 20 mg/L resulted in the percentage of shoot-buds regeneration significantly decreased (Table 2). Hence, the optimum TDZ concentration for adventitious buds induction was 20 mg/L.

Table 2. Result of dealing with hypocotyl explants by different concentrations of TDZ solution for inducing adventitious buds regeneration in *J. curcas* after 30 days of culture.

TDZ solution concentration(mg/L)	Regeneration percentage (%)	Number of buds per explant
0	0d	0e
10	52.45±4.24c	4.19±0.62d
20	81.91±2.65a	10.16±0.81a
30	64.44±3.85b	7.48±0.34b
60	52.96±3.14c	5.33±0.30c

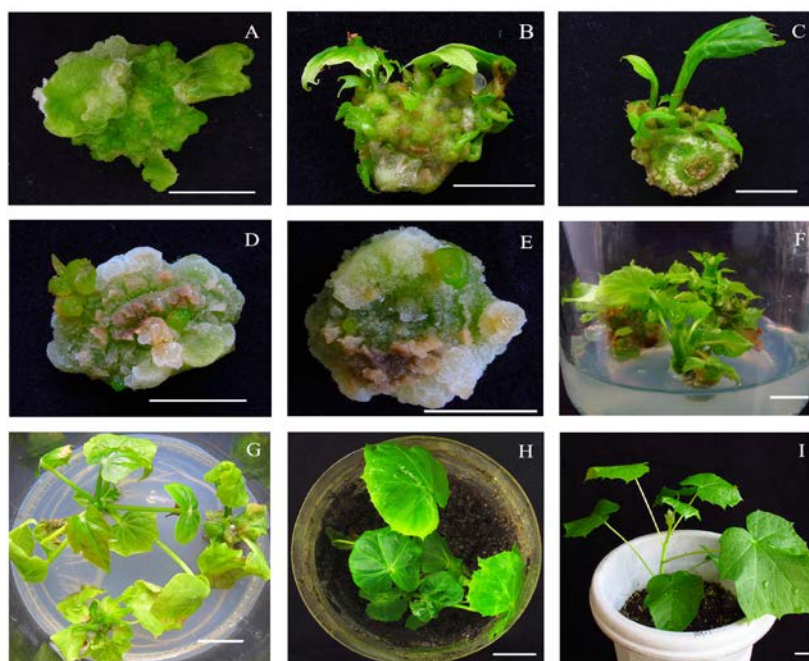


Fig. 1. Induction of shoot-buds, elongation and rooting of regenerated buds from hypocotyl explants in *J. Curcas*.

(A) Hypocotyl explants were placed onto MS medium supplemented with 0.6 mg/L TDZ for 30 days; hypocotyl explants *in vitro* were inoculated horizontally (B) and vertically (C), and *in vivo* hypocotyl explants were placed horizontally (D) and vertically (E) on hormone-free MS medium for 30 days after being dealt with 20 mg/L TDZ solution for 20 min; (F) Elongation of regenerated adventitious buds in MS medium contained 0.25 mg/L KT, 0.5 mg/L 6-BA, 0.25 mg/L IAA and 0.4 mg/L GA3 for 15 days; (G) a regenerative plant in 1/2 MS medium included 0.3 mg/L IBA and 16 mg/L Gln for 20 days; (H) a intact plant acclimatization in plastic bottle for 10 days and (I) in a pot for further growth and development for 50 days. (bar =1 cm)

For purpose of investigating the effect of duration of TDZ solution treatment on shoot-buds induction, hypocotyl explants were dealt with 20 mg/l TDZ solution for different period of time previous to inoculate explants onto hormone-free MS medium. When the explants were dealt with 20 mg/L TDZ solution for 5 min before culture, the induction percentage of regenerated buds was 53.61%, and the number of shoot-buds per explants was 5.46 (Table 3). This result was superior to the best consequence gained via applying general culture protocols (Table 1). Dealing with the

explants with 20 mg/L TDZ solution for 20 min was the optimum condition and it acquired the highest percentage of regeneration (81.91%) and the maximum number of regenerated shoot-buds per explant (10.16) (Table 3 and Fig. 1B). When the explants were dealt with 20 mg/l TDZ for time of duration more than 20 min, i.e. 30 and 60 min, the frequency of shoot regeneration was reduced markedly; the percentage of adventitious buds induction was altered from 61.51% to 72.36%, and the quantity of induced shoot-buds per explant was varied from 6.99 to 8.25 (Table 3).

Table 3. Result of dealing with explants with TDZ solution for different durations on shoot-buds regeneration from hypocotyl explants of *J. curcas* after 30 days of culture.

Treatment duration (min)	Regeneration percentage (%)	Number of buds per explant
0	0d	0d
5	53.61±3.37d	5.46±0.52d
20	81.91±2.65a	10.16±0.81a
30	72.36±3.48b	8.25±0.47b
40	61.51±4.81c	6.99±0.32c

Comparison the Regeneration Effect of Inoculation Methods on Culture

For the sake of study the effect of inoculation orientation (horizontal or vertical) on shoot-buds induction, the hypocotyl explants were inoculated onto hormone-free MS medium horizontally or vertically after being treated with 20 mg/L TDZ for 20 min. The results of our study showed distinctly that the way of placing the explants horizontally on the medium for regeneration was much more advantageous than inoculating the explants vertically (Table 4, Fig. 1B-D). The number of induced adventitious buds per explant was altered from 2.04 to 10.16 in the horizontal position and 1.17 to 4.27 in the vertical position, whilst the percentage of adventitious buds induction was varied from 25.70 to 81.91% in the horizontal position and 11.03 to 38.51% in the vertical position (Table 4, Fig. 1B-D).

Table 4. Results of the explants orientation on the regeneration of shoot-buds from hypocotyl explants in *J. curcas* after 30 days of culture.

Explant source	Orientation	Regeneration percentage (%)	Number of buds per explant
<i>In vitro</i>	Horizontal	81.91±2.65a	10.16±0.81a
	Vertical	38.51±3.40b	4.27±0.45b
<i>In vivo</i>	Horizontal	25.70±2.59a	2.04±0.25a
	Vertical	11.03±1.81b	1.17±0.11b

Results of L-glutamine (Gln) on rooting of shoots

Though the elongated shoot-buds could be induced rooting in the MS medium containing 0.3 mg/L IBA, the rooting efficiency was comparatively low, and the highest rooting induction efficiency was 23.68% (Table 5 and Fig. 1F). Our results indicated that the activation and growth of roots were stimulated effectively attributing to supplement with Gln at specific concentrations into the mediums, and the greatest rooting rate (51.39%) was obtained when 16 mg/L Gln having been applied into MS medium contained 0.3mg/L IBA in the present study (Table 5). However, the regeneration of roots was inhibited significantly when the concentrations of added Gln was exceed 16 mg/L (Table 5 and Fig. 1G). When the elongated shoos were inoculated into the MS medium containing only 16 mg/L Gln, the rooting rate of the elongated shoot buds was merely 11.14% at 40 days of culture (Table 5).

Regenerated plantlets were acclimatized in plastic bottle for 15 days (Fig. 1H) and then transplanted successfully into the soil for normal growth (Fig. 1I).

Table 5. Results of different L-glutamine concentrations on the rooting of regenerated shoots from hypocotyl explants in *J. curcas*.

Culture time (days)	Gln concentration (mg/L)	Rooting percentage (%)	Number of roots per shoot	Average root length (cm)
10	0	8.36±2.44d	1.27±0.25b	0.65±0.23b
	2	9.17±2.86d	1.33±0.29b	0.68±0.15b
	8	23.77±4.25b	2.25±0.25a	0.92±0.24b
	16	31.25±3.24a	2.35±0.36a	1.33±0.19a
	24	21.93±2.98bc	2.09±0.23a	1.27±0.18a
	48	16.90±2.03c	1.32±0.33b	0.91±0.10b
	CK1*	0e	0c	0c
	CK2**	5.63±2.05d	1.33±0.58b	0.65±0.10b
20	0	15.32±1.22c	3.11±0.19c	1.61±0.14c
	2	16.59±5.28c	3.31±0.34c	1.66±0.15c
	8	32.63±4.52b	4.39±0.41b	2.42±0.25b
	16	38.16±4.63a	5.11±0.28a	2.92±0.32a
	24	30.98±2.14b	4.29±0.29b	2.56±0.27ab
	48	18.57±1.54c	3.25±0.43c	2.37±0.24b
	CK1	0e	0e	0d
	CK2	7.08±2.22d	1.50±0.5d	1.33±0.23c
30	0	22.09±2.98d	3.57±0.40d	3.37±0.31c
	2	24.12±3.40d	3.85±0.51d	3.50±0.29bc
	8	35.24±3.56bc	4.99±0.61c	4.01±0.30b
	16	49.94±2.09a	5.91±0.31a	4.77±0.43a
	24	39.77±4.36b	5.36±0.21ab	4.63±0.29a
	48	30.83±2.21c	3.90±0.63d	3.53±0.35bc
	CK1	0f	0f	0e
	CK2	9.97±1.81e	1.67±0.29e	1.78±0.18d
40	0	23.68±1.39d	4.53±0.42d	3.75±0.50c
	2	25.87±2.16d	4.73±0.64d	3.83±0.45c
	8	36.75±4.97c	5.61±0.23bc	4.51±0.26b
	16	51.39±1.21a	6.43±0.25a	5.37±0.39a
	24	42.81±4.36b	5.89±0.18ab	5.03±0.21ab
	48	32.50±3.01c	4.93±0.44cd	3.86±0.30c
	CK1	0f	0f	0e
	CK2	11.44±2.52e	1.72±0.25e	1.82±0.19d

*CK1: hormone-free MS medium; **CK2: MS medium contained 16 mg/L Gln.

Discussion

Direct inoculation of hypocotyl explants onto mediums containing cytokinin such as TDZ at low concentrations (usually 0.05-1 mg/L) is the conventional culture protocols for inducing regeneration of shoot-buds in *J. curcas* [20-23]. However, the conventional methods showed low regeneration efficiency and the shoot-buds were hard to elongation sequentially. The regeneration frequency and the formation of bigger buds were facilitated due to apply the method of dealing with hypocotyl explants with high concentration of TDZ solution for a short time before placing onto hormone-free MS medium. Furthermore, then continued to culture the regenerated buds revealed that those

shoot-buds were readily elongated.

Cytokinin was a key factor for adventitious buds induction in plant tissue culture universally [14, 19, 28, 29]. TDZ was a plant growth regulator belonged to the cytokinin family. Our results indicated that cytokinin might be needed for a short time in the process of induction of adventitious buds after treatment with high concentration of TDZ solution. Nevertheless, it's unclear that how TDZ played a part in inducing of adventitious buds in our protocols. Our previous research found that the frequency of adventitious buds regeneration from hypocotyl explants was increased markedly with high concentrations of BA solution treatment before inoculating the explants on hormone-free MS medium in soybean [30]. These results implied that although various cytokinins should be detected for optimum results, our culture protocols could be large-scale applied to other plant species.

IBA was subject to the auxin family and deemed to be contributing in the rooting of *J. curcas* definitely [9,31-33]. The rooting efficiency was relatively low when the shoots were induced to rooting in the MS medium just included 0.3 mg/L IBA. Gln was regarded as a endogenous amino acids, which supplied nitrogen for the biosynthesis of nucleic acids and other N-compounds and participated in plant metabolism [34]. Therefore, Gln was used in mediums as an organic nitrogen source frequently [35]. Some reports indicated that exogenous Gln might have some beneficial influenced on rooting of shoots in numerous plants [36-38]. Our study results suggested that the efficiency of shoots rooting could be improved dramatically attributing to applying the exogenous Gln in *J. curcus*.

In the present study, the proliferation and elongation of shoots were implemented synchronously, and then the period for obtaining intact plantlets was cut down signally without compromising the quality and quantity of regenerated buds. On account of the modified shoots elongation and efficient roots induction, an intact plantlet could be acquired in 60 days of culture via applying the new protocol described above, while there were time-consuming by conventional methods needed 90-140 days of culture [20,23].

Conclusion

In short, a high efficiency *in vitro* culture method for achieving regenerated plantlets was founded in the present study. This efficient and reproducible protocol would be beneficial for increasing quantity of regenened plants and could be applied to generating transgenic plants via *Agrobacterium* mediated transformation.

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