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Direct Shoot Organogenesis from Juvenile Cotyledon of *Jatropha podagrica* Hook

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ABSTRACT

Jatropha podagrica Hook is one species of the genus Jatropha. The Roots and vegetative parts of J. podagrica contain biologically active compounds and its seeds comprise high oil content. There is urgent need to develop mass propagation of J. podagrica by in vitro cultures. The objective of this research was therefore to induce direct shoots from juvenile cotyledon. Seeds were grown in vitro for 12 days and juvenile cotyledons excised for direct shoot organogenesis. MS (Murashige & Skoog) solid medium supplemented with 2.22-13.32 µM BAP, 0.49 µM IBA and 2.27-6.81 µM TDZ enriched with 3% (w/v) sucrose and 0.8% agar were used. The best shoot induction (3.00 ± 0.89 shoots per explant) was achieved after 6 weeks at a combination of 2.22 µM BAP, 0.49 µM IBA and 4.54 µM TDZ. The elongated renewed shoots were transferred to root induction medium containing half strength MS salts supplemented with either indole 3-butyric acid (IBA) or indole acetic acid (IAA). Best rooting was achieved on the medium supplemented with 9.8 µM IBA. Rooted plantlets were then acclimatized under greenhouse and showed 60% survival rate.

Keywords: *Jatropha podagrica*, Juvenile cotyledon, Shoot induction, Acclimatization, Micropropagation

INTRODUCTION

Jatropha podagrica is a shrub commonly known as coral nut, Guatemala Rhubarb and physic nut; and it belongs to family Euphorbiaceae. It is a tropical America innate, but similarly common in southern part of Africa, Australia and warmer portions of Asia [1]. Jatropha originate from "jatros" the Greek word which means doctor also "trophe" food, that suggests the therapeutic practises [2]. *J. podagrica* under the tribe Joannesieae of the family Euphorbiaceae comprises about 165-170 identified types worldwide primarily in tropical and subtropical areas. Jatropha is a tremendously adaptive plant thus it can grow in dry lands and degraded soils. *J. podagrica* is a very significant plant. It is a rich source of biologically active compounds, used for antimicrobial activities. It is also a famous ornamental plant and most remarkably, a promising biodiesel plant. Some of the many secondary metabolites extracted from *J. podagrica* are scoparone, 3-Acetylaleuritolic acid [1], fraxidin, japodic acid and erythrinasinate [3]. Seed oil content varies among species from 30-38%. Farming of the Jatropha plant is a promising source of renewable energy (biodiesel oil). Some nontoxic varieties can also offer edible oil for human consumption. Moreover, kernel slab for cattle [4]. Sujatha et al. [5] reported old-style proliferation done by stem cuttings as possible. Nevertheless, it is periodic and vulnerable to diseases. In plant biotechnology, induction of callus and regeneration are crucial tools that exploit the totipotency of a plant cell. In the last two decades, successful indirect (callus mediated) *in vitro* regeneration of *J. curcas* has been reported. It is therefore necessary to investigate these techniques for mass reproduction of *J. carcus* to

guarantee an easy cultivation of this leading substantial plant [6-8]. Shoots can be encouraged both by differentiation (adventitious bud launch) and present meristematic active growing tissues such as axillary bud establishment. *In vitro* renewal is influenced by kind and developmental stage of the explants, medium formulation, hormone combination and concentration of gelling agents [5,7,9]. Plant growth and development are regulated by growth hormones. Hormones control speed of development of distinct parts then assimilate these portions to yield plants. PGRs either auxins or cytokinins alone or in combination are required to persuade cell division and differentiation [7]. Many researchers agree that dissimilar mixtures of cytokinins such as 6-benzyladenine (BA), kinetin (Kn) and auxins such as NAA, IAA etc. control straight adventitious manifold shoot bud regeneration from various explants such as petiole, hypocotyl, and cotyledon of *J. curcas* [5,7,9,10].

Jatropha can be easily propagated by conventional means and highly cross pollinated but variations among species are limited and heterozygous seeds that decreases oil yield. Moreover, the method is seasonal [11]. Therefore, an efficient *in vitro* Propagation technique is vital to harvest healthy, uniform and rapid clonal propagates whenever extraction of secondary metabolites for medicinal use and renewable biodiesel oil is needed. In this study, *in vitro* grown juvenile cotyledon was used as an explant to develop a direct shoot organogenesis of *Jatropha podagrica* for subsequent use in mass cultivation and *in vitro* clonal propagation. Different hormone combinations and concentrations were compared for their effect in the initiation of shoots and roots.

MATERIALS AND METHODS

Plant source

J. podagrica seeds were obtained from the greenhouse, Faculty of Agriculture, Naresuan University, Thailand. Seed coats were removed and seeds were disinfected by soaking in 25% sodium hypochlorite for 10 min and rinsed three times with autoclaved distilled water to remove the traces of sodium hypochlorite. Treated decoated seeds were inoculated on MS free solid medium to get the juvenile cotyledon explants for bud initiation.

Shoot bud induction

The juvenile cotyledon gained from *in vitro* seedling grown for 12 days were refined on MS basal salts. Medium was complemented with various concentrations and combinations of PGRs viz 2.22-13.32 µM BAP, 0.49 µM IBA and 2.27-6.81 µM TDZ to discover the optimum and suitable medium for direct shoot induction [12]. One explant (juvenile cotyledon) per media combinations was positioned on medium surface in 4-ounce bottles. Development of buds were noted and recorded after six weeks. Experiments were repeated twice to establish reliability of the obtained results.

Shoot elongation

Juvenile cotyledon initiated shoot buds were transferred to shoot elongation medium supplemented with 4.65, 6.98 µM kinetin (Kn), 4.44-6.66 µM 6-benzyle adenine purine (BAP) and 1.43, 2.86 µM indole -3-acetic acid (IAA) [13], individually or in combination. Shoot length data were collected after one month.

Rooting and acclimatization

Juvenile cotyledon derived elongated shoots were moved to half strength MS for rooting. Sucrose concentration of the media was reduced from 3% to 2% and gelled with 0.8% agar prior to autoclaving. The medium was further enriched with 4.9-9.8 µM IBA or 5.71-17.13 µM IAA. Rooted plantlets were moved to plastic vessels after 4 weeks. Plant roots were washed thoroughly with autoclaved distilled water to eliminate the adhering media traces prior to transfer in plastic pots containing farmyard manure. Pots were covered by plastics with small holes for aeration to prevent water loss by transpiration and incubated in the greenhouse.

Culture conditions and data analysis

Throughout the study, the medium used for culture and sub culture was MS (Murasige & Skoog). In all experiments, media pH was adjusted to 5.8 using either 1 N NaOH or HCl prior autoclaved. The same media strength (full strength) was used to initiate shoot bud and shoot elongation. On the other hand, half strength MS medium was used for root induction. Full strength media was added with 3% sucrose as carbon source whereas half strength MS medium was enriched with 2% (w/v) sucrose. In all experiments media was gelled with 0.8% (w/v) agar and sterilized by autoclaving at 12°C and 15 psi pressure for 20 min. After inoculation under aseptic techniques all cultures were kept in tissue culture chamber at 25 ± 2°C under a 12 h photoperiod rotation with light strength of 35-40 µmol m^{-2} s^{-1} (cool white fluorescent tubes).

Trials were established in a totally randomized experimental design. Each treatment was done in six culture bottles in two replication trials. Homogeneity among and within treatments was tested by Duncan's multiple range test using SPSS version 19. The outcomes are articulated as the means ± SD of two independent tests. Moreover, data were exposed to one way-ANOVA test.

RESULTS AND DISCUSSION

In vitro seed germination

Like all Jatropha species seed of *J. podagrica* display poor response to germination due to its impermeable seed-coat. Jatropha can be propagated by conventional method and highly cross pollinated but still have limited genetic variation. *In vitro* mass multiplication by intra-varietal hybridization using the young embryos was reported [11]. In the study, husk was removed and seeds inoculated after which germination started between the 5th and 8th days when refined on MS free solid medium. The benefit of seed coat removal for quick and simple germination has been stated in regeneration study of *Embelia ribes* [14]. Encouraging effect of physiological pre-sawing treatments in breaking seed inactivity and improving germination proportion have also been reported [15]. When the endosperms started to open and radicle emerge, the juvenile cotyledons were removed and refined on MS medium enhanced with growth hormones.

Shoot induction from juvenile cotyledon of *Jatropha podagrica*

The *in vitro* response of *J. podagrica* juvenile cotyledon to various media composition and concentration is illustrated in Table 1. Depending on the media composition average shoot number ranging from 1 ± 0.00 to 3 ± 1.00 were established at the cut ends of juvenile cotyledon. Shoot induction opportunity from juvenile cotyledon appeared to be important in the occurrence of PGRs. Whereas, MS free did not encourage any morphogenic differences on the refined juvenile cotyledons. The excised juvenile cotyledons responded to various hormone combinations by increasing size on an average five folds of the original size and curling, after one week from the date of culture (Figures 1A-1C). Subsequently, thin calli layers were started within the second week from the date of culture. Noticeable induction of calli were observed at the cut edges predominantly on the media accompanied by 2.22-13.32 µM BAP and 0.49 µM IBA. On the other hand, all cultures fortified with 2.22, 4.44 µM BAP, 0.49 µM IBA and 2.27, 4.54 µM TDZ provide direct shoot induction as indicated in Figures 1D and 1E). The various hormone combinations and concentrations used also revealed a significant effect on shoot growth. Cotyledons refined on MS added with 2.22 µM BAP, 0.49 µM IBA and 4.54 µM TDZ showed fully developed shoots (Figure 1D). However, there was an intervening callus development on the explant along with shoot bud growth from the cut edges. In our study, TDZ played a vital role in regenerating shoots. All explants cultured on media combinations without TDZ hormone converted to callus. Increasing concentration of TDZ from 2.27 µM to 6.81 µM in the absence of IBA lowers the rate of shoot bud induction from 2.00 ± 1.09 to 1.00 ± 0.00 (Table 1). The result showed that the effect of TDZ hormone in shoot induction was influenced by IBA. Khemkladngoen et al. [12] reported that all plant growth hormones with or without TDZ hormone induce shoots from a juvenile cotyledon explant of *J. curcas*. In their study, the uppermost incidence of shoot renewal was empirical at a combination of 13.32 µM BAP and 0.49 µM IBA and combination of 2.22 µM BAP, 0.49 µM IBA and 2.27 µM TDZ. Contrastingly, in our study, cultures treated with 2.22-13.32 µM BAP and 0.49 µM IBA induce a noticeable callus but did not produce shoot at all. The combinations and concentrations of 2.22, 4.44 µM BAP, 0.49 µM IBA and 2.27 µM TDZ promoted direct shoot induction and the highest number of shoots (3.00 ± 0.89) was attained at this combination. Our result considerably agrees with the finding [12], in the second hormone combination.

Shoot elongation

The maximum elongation of regenerated shoots was 2.03 ± 0.07 cm (Table 2) obtained from MS media enriched with 6.98 µM Kn or 6.66 µM BAP and 1.43, 2.86 µM IAA. Both cytokinin Kn and BAP at the same concentration demonstrated the same effect and there was no statistically measurable difference of the increased IAA concentration from 1.43 µM to 2.86 µM. Numerous buds were noticed at a combination of 2.22 µM BAP, 0.49 µM IBA and 4.54 µM TDZ (Figure 1E). However, when these shoot buds are moved to another media (MS+Kn+BAP and IAA) for elongation it only increased in length without primary shoots as shown in Figure 1F. On the other hand, microshoots inducted from a combination of 2.22 µM BAP, 0.49 µM IBA and 4.54 µM TDZ and transferred on the same elongation medium showed significant elongation with primary shoot development (Figure 1G).

Rooting and acclimatization

Plentiful rooting development of plantlets under *in vitro* situations is significant for fruitful establishment of the

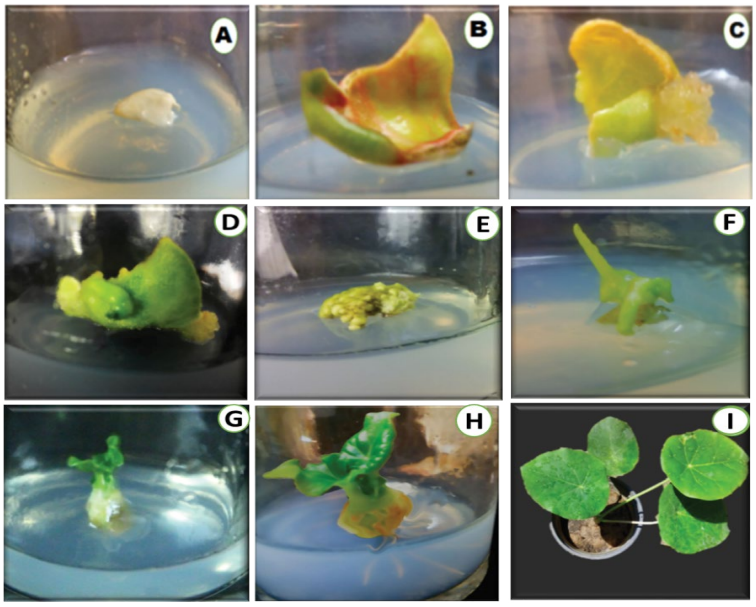

Figure 1: Shoot induction from juvenile cotyledon of *J. podagrica.* **A:** Juvenile cotyledon explant in the first day of culture. **B:** Curling and size increase 4-5 folds after one week. **C:** Calli induction from cotyledon explant cultured on MS+BAP (4.44, 8.88, 13.32 µM)+IBA (0.49 µM). **D:** visible shoot buds at the edges on MS+BAP (2.22 µM)+IBA (0.49 µM)+TDZ (4.54 µM). **E:** Numerous but small buds on MS+BAP (2.22 µM)+IBA (0.49 µM)+TDZ (2.27 µM). **F,G:** shoot elongation on (MS+Kn+BA & IAA). **H:** Rooting on half strength MS+IBA. **I:** Acclimatized plant in green house

Table 1: Effect of several combinations of BAP, IBA and TDZ on shoot induction from juvenile cotyledon of *J. podagrica*

Treatment	BAP (µM)	IBA (µM)	TDZ (µM)	Callus Formation (+)	Shoots/Explant
1	0	0	0	(-)	-
2	2.22	0.49	2.27	(-)	2.33 ± 0.51^{ab}
3	2.22	0.49	4.54	(-)	3.00 ± 0.89^{a}
4	4.44	0.49	2.27	(-)	2.00 ± 1.09^{bc}
5	4.44	0	2.27	(-)	2.00 ± 0.00^{bc}
6	4.44	0	4.54	(-)	1.33 ± 0.51^{bcd}
7	8.88	0	6.81	(-)	1.00 ± 0.00^{d}
8	4.44	0.49	0	(+)	-
9	8.88	0.49	0	(+)	-
10	13.32	0.49	0	(+)	-

The same later indicate no significant difference (p<=0.05)

Table 2: The effect of MS basal medium supplemented with Kn, BA, IAA on shoot elongation

Treatment	Kn (µM)	BAP (µM)	IAA (µM)	Shoot Length (cm)
1	0	0	0	0.71 ± 0.18^{e}
2	4.65	0	0	1.36 ± 0.25^{d}
3	6.98	0	0	1.49 ± 0.10^{cd}
4	0	4.44	0	1.59 ± 0.12^{c}
5	0	6.66	0	1.74 ± 0.05^{b}
6	6.98	0	1.43	1.92 ± 0.13^{a}
7	6.98	0	2.86	2.01 ± 0.15^{a}
8	0	6.66	1.43	2.03 ± 0.05^{a}
9	0	6.66	2.86	2.03 ± 0.07^{a}

The same letter indicate no significant difference (p<=0.05)

regenerated plants [16]. The *in vitro* propagated shoots were able to iniciate roots on half strength MS fortified with either IBA or IAA. Root number varies from 1 to 5 depending on the concentration of PGRs and achieved within 20-25 days from the date of culture. Despite medium accompanied with 300 mg/l activated charcoal, there was low level of browning at the cut edges of the shoots during rooting. Microshoots cultivated on media enhanced with 4.9, 7.35, 9.8 µM IBA established 2-5 roots within 20-22 days of culture (Figure 1H) and the maximum root number

Table 3: Effect of half strength MS basal medium supplemeted with IBA, IAA on rooting of *in vitro* induced shoots

Treatment	IBA (µM)	IAA (µM)	root number
1	0	0	0.75 ± 0.5^{c}
2	4.9	0	2.5 ± 0.57^{b}
3	7.35	0	3.25 ± 0.95^{b}
4	9.8	0	4.25 ± 0.50^{a}
5	0	5.71	1.00 ± 0.00^{c}
6	0	11.42	1.50 ± 0.57^{c}
7	0	17.13	1.75 ± 0.50^{c}

The same letter indicate no significant difference ($p<=0.05$)

4.25 ± 0.5 (Table 3) was noted in the medium comprising half strength MS suplemented with 9.8 µM IBA. Root induction was low in medium complemented with IAA and comperatively took longer time (25 days). The efficiency of IBA in rooting microshoots has been reported in a number of plants including *J. curcas* [5,16]. A significant rise in root number (2.5 ± 0.57 to 4.25 ± 0.50) was observed as the concentration of IBA increased from 4.9 µM to 9.8 µM while, there was little rooting effect in increased IAA and there was no measurable difference between MS free and MS complemented with IAA. Rooted plantlets were carefully removed from the culture bottles and washed with autoclaved distilled water before transferred to pots filled with farmyard manure and kept for one month under the shade in greenhouse. Development of new shoots was visible after three weeks of transfer with 60% survival rate (Figure 1I).

A simple and efficient *in vitro* plant regeneration through direct organogenesis protocol was established for *J. podagrica* using juvenile cotyledon explants. the full regeneration of the plant (seed germination to plantlet) was acomplished within 4 months and 3 weeks. This technique can be used for subsequent mass propagation of true-to-type plants and genetic transformation of the substantial biofuel plant.

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