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Adenine sulfate and glutamine enhanced shoots multiplication in *Jatropha curcas* L. a potent biofuel plant

Ismail A. Mohammed[1]*, Suguru Tsuchimoto[2], Hiroe Sakai[2], Naoki Wada[3] and Kiichi Fukui[2]

[1]Department of Botany and Agricultural Biotechnology, Faculty of Agriculture, University of Khartoum, Khartoum, Sudan.
[2]Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan
[3]Graduate School of Technology, Industrial and Social Sciences, Tokushima University, Tokushima 770-0814, Japan.

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Jatropha is a drought-tolerant plant producing seed oil for the biodiesel. Limitation to the development of jatropha is unavailability of high-yielding varieties and efficient *in vitro* regeneration system which is required for micropropagation. In this study, *in vitro* regeneration system from jatropha juvenile cotyledon was established. Firstly, concentrations of hormones in the MS medium were optimized and it was found that 1.5 mgL^{-1} benzyl adenine (BA) + 0.05 mgL^{-1} indole-3-butyric acid (IBA) + 0.5 mgL^{-1} thidiazuron (TDZ) turned out to be the best for shoot induction (3.82 ± 0.18 shoots/explant). Secondly, shoot induction medium was fortified with different concentrations of glutamine and adenine sulfate. It was found that 25 mgL^{-1} each of glutamine and adenine sulfate was the most effective, resulting to 9.09 ± 0.37 shoots/explant and 93.0% regeneration frequency. Regenerated shoots were cultured on medium containing 0.5 mgL^{-1} BA and different concentrations of gibberellic acid (GA_3), 0.5 mgL^{-1} GA_3 with 0.5 mgL^{-1} BA were found to be the best for shoot elongation (2.13 ± 0.18 cm). The highest frequency of root (40%) was observed on the medium with 0.5 mgL^{-1} IBA. The established procedures will be useful for the mass propagation and genetic transformation of elite jatropha genotypes.

Key words: Adenine sulfate (Ads), benzyl adenine (BA), indole-3-butyric acid (IBA), *in vitro* regeneration, glutamine (Gln)

INTRODUCTION

Jatropha *(Jatropha curcas*) is a potent biofuel crop native to Mexico and Central America and now is distributed throughout tropical and subtropical regions (Kumar and Tewari, 2015). Its seed contains high amount of oil in the concentration of 30 – 60% (Openshaw, 2000; Kumar and Sharma, 2008), which is non-edible (Chhetri et al., 2008) and suitable for the biodiesel (Heikal et al., 2015). Due to the depletion of fossil fuel reserves, increasing petroleum prices and global climate changes, jatropha has received considerable attention as renewable energy sources

*Corresponding author. E-mail: ismaildamer@mail.com.

(Pandey et al., 2012). However, the low productivity under certain conditions restricts fuel usage of jatropha, mainly because it has not been domesticated for large-scale production. Therefore, increasing oil yield, growing ability under abiotic stresses and improvement of agronomic traits must have a priority. Jatropha plant is propagated through asexual methods and by seeds, and thus seed yield and oil content varies significantly (Jha et al., 2007). Furthermore, seeds of *J. curcas* have a limited viability and can only be stored for 15 months after which their viabilities are reduced by 50% (Kochhar et al., 2005). Cuttings propagation of jatropha can be carried out for maintaining true to type genotypes, but the produced plants do not have deep roots and the quality is not sufficient to meet the growing demand of *J. curcas* (Heller, 1996; Openshaw, 2000). Additionally, propagated plants exhibited lower longevity and resistance to drought and diseases (Sujatha et al., 2005). Generally, vegetative propagation methods have drawbacks such as sources of disease transmission (Fufa et al., 2019). Therefore, an efficient *in vitro* regeneration for mass production of disease free and true to type jatropha genotypes is desirable. It can propagate superior genotypes and contributes to plant improvement through the application of biotechnological techniques.

Recently, several studies have been reported on the regeneration of jatropha by using various explants and different combinations of phytohormones and additives (Chiangmai et al., 2015; Gangwar et al., 2015; Jadon et al., 2015; Liu et al., 2015; Mishra, 2018; Fufa et al., 2019). BA and IBA were found to be effective growth regulators for the induction of callus and shoot regeneration from various explants of *J. curcas* plant. Previously in the laboratory, the suitable concentration of TDZ (0.5 mgL^{-1}) for callus induction and combination of BA and IBA for shoot multiplication from juvenile of jatropha were established by Khemkladngoen et al. (2011). However, the low regeneration efficiency is a main obstacle to jatropha regeneration. Thus, this study—aimed to investigate the effect of adding adenine sulfate and glutamine in shoot regeneration media on shoots multiplication. Here, we have developed an efficient *in vitro* regeneration protocol for shoots induction, multiplication and plant regeneration from juvenile cotyledons explants.

MATERIALS AND METHODS

All experiments were conducted at Plant Bioengineering for Bioenergy Laboratory, Department of Biotechnology, Osaka University. Suita City, Osaka, Japan.

Plant materials and preparation of explants

Mature decorated seeds of jatropha (Thai line) were surface-sterilized with 70% (v/v) ethanol for 2 min followed by 40% (v/v) sodium hypochlorite and 0.01% Triton X-100 for 10 min, and then washed five times with sterile distilled water. After washing, surface-sterilized seeds were soaked overnight. The sterilized seeds were germinated *in vitro* on half strength hormone-free Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose, 10 mgL^{-1} thiamine, 100 mgL^{-1} *myo*-inositol (pH 5.8) and 0.8% agar at 25°C for one week. The juvenile cotyledons (Figure 1a) were used to prepare explants as described in Khemkladngoen et al. (2011). They were cut into pieces (3 mm x 3 mm) and used as explants (Figure 1b).

Callus induction

Explants were cultured on a callus induction medium consisting of MS medium supplemented with different concentration sets of benzyl adenine (BA: 1.0, 1.5 and 3.0 mgL^{-1}) and indole-3-butyric acid (IBA: 0.5 and 0.05 mgL^{-1}), as well as 0.5 mgL^{-1} thidiazuron (TDZ) and 0.8% (w/v) agar. Cultures were incubated at 25 ± 2°C under 16-h lights (31-35 µmol photon m^{-2} s^{-1})/ 8 h dark photoperiod for two weeks. The experiment was replicated twice.

Shoot regeneration

Two approaches were used in this study. Firstly, calli induced from cotyledon explants in the callus induction media were subcultured on MS medium supplemented with the same concentration sets of BA and IBA as the callus induction media. Secondly, calli induced from cotyledon explants in the callus induction medium containing 1.5 mgL^{-1} BA, 0.05 mgL^{-1} IBA and 0.5 mgL^{-1} TDZ were subcultured on MS medium supplemented with combination of 1.5 mgL^{-1} BA and 0.05 mgL^{-1} IBA in the presence of different concentrations of adenine sulfate and glutamine mixture (0, 5, 10, 15, 20 and 25 mgL^{-1} each). Cultures were incubated under the same condition as described above for 2 weeks and subcultured twice. The frequency of shooting response and shoots formed per explant were recorded. Each experiment was conducted twice.

Shoot elongation

The regenerated shoots were cultured on MS medium supplemented with 0.5 mgL^{-1} BA in combination with different concentrations (0.1 – 1.0 mgL^{-1}) of gibberellic acid (GA_3) for shoot elongation. Cultures were incubated under the same condition as described above for 4 weeks. The experiment was replicated twice.

Rooting and acclimatization

The elongated shoots were excised individually and transferred to rooting medium, which consisted of a half-strength B5 medium supplemented with 2% (w/v) sucrose, 10 mgL^{-1} thiamine, 100 mgL^{-1} *myo*-inositol, four concentrations of glutamine (0, 5, 10 and 15 mgL^{-1}) in combination with 0.5 mgL^{-1} IBA and 0.7% agar (pH 5.8). Cultures were incubated under the same condition as described above for 6 weeks. Plantlets with rooted shoots were transplanted into autoclaved soil in small pots covered with transparent plastic lids and maintained under high humidity for 7 days, and thereafter gradually exposed to the growth chamber condition. Established plantlets were then transferred to plastic pots containing soil and cultivated in a growth chamber. Each experiment was replicated twice.

Statistical analysis

Data of shoot number per explant, percentage of explant response,

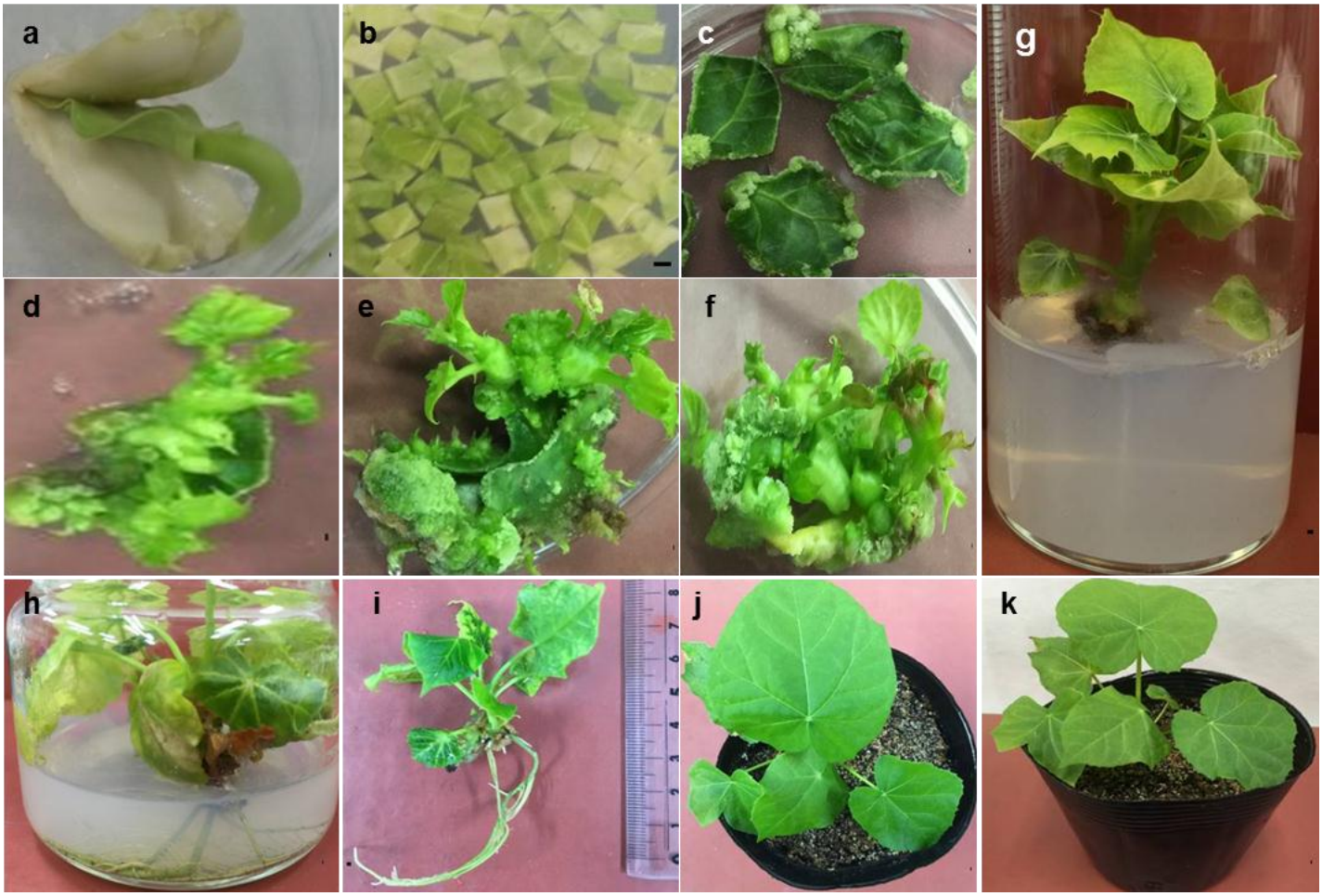

Figure 1. *In vitro* regeneration of jatropha, a) Juvenile cotyledons used as explants cultured on ½ MS basal medium; b) Incised cotyledons cultured on MS medium supplemented with 1.5 mgL^{-1} BA, 0.05 mgL^{-1} IBA and 0.5 mgL^{-1} TDZ at 0 day; c) Callus induction on MS medium supplemented with 1.5 mgL^{-1} BA, 0.05 mgL^{-1} IBA and 0.5 mgL^{-1} TDZ after 15 days; d) Shoot initiation on MS medium supplemented with 1.5 mgL^{-1} BA and 0.05 mgL^{-1} IBA after 30 days; e) Shoot initiation on MS medium supplemented with 1.5 mgL^{-1} BA, 0.05 mgL^{-1} IBA and 25 mgL^{-1} Gln and Ads after 15 days; f) Shoot multiplication on MS medium supplemented with 1.5 mgL^{-1} BA, 0.05 mgL^{-1} IBA and 25 mgL^{-1} Gln and Ads after 30 days; g) Shoot elongation on MS medium supplemented with 0.5 mgL^{-1}BA and 0.5 mgL^{-1} GA_3 after 30 days; h and i) Rooted plantlet in ½ B5 medium supplemented 0.5 mgL^{-1} IBA after 45 days; j and k) Plantlets after acclimatization. Bars = 1 mm.

shoot length, root number per explant and percentage of root shoot were collected. Recorded data were analyzed using one-way ANOVA and the mean separations were carried out using Tukey's HSD test at $P \leq 0.05$. All statistical analysis was performed using SPSS 22.0 (SPSS Inc. USA).

RESULTS AND DISCUSSION

Shoot induction and multiplication

The results showed that BA and IBA affected the shoot regeneration with 0.5 mgL^{-1} TDZ, and the combination of 1.5 mgL^{-1} BA and 0.05 mgL^{-1} IBA was the most effective in regenerating shoots from calli (3.82 ± 0.18 shoots/explant) (Table 1 and Figure 1c, d). This finding was similar to previous results reported by Chiangmai et al. (2015), Nanasato et al. (2015) and Fufa et al. (2019). The study was divergent from previous study in our lab by Khemkladngoen et al. (2011) which showed that the combination of 3 mgL^{-1} BA and 0.1 mgL^{-1} IBA produced the highest regeneration frequency from calli due to genotype difference.

Low shoot multiplication rate is a major constraint facing *in vitro* regeneration protocol of jatropha plant. Evaluation on the synergistic effect of glutamine and adenine sulfate on shoot regeneration and multiplication was further studied. The effectiveness of organic nitrogen source particularly glutamine for multiplication and maintenance of healthy *in vitro* tissue for long time periods have been reported in other plant species (Green et al., 1990; Ogita et al., 2001; Vasudevan et al., 2004; Sanjaya et al., 2005). The synergistic effects of adenine

Table 1. Effect of different combinations of BA and IBA on shoot regeneration from juvenile cotyledon

Concentration (mgL^{-1})*		Number of shoots/ explant**
BA	IBA	
1.0	0.5	2.50 ± 0.18^{ab}
1.0	0.05	2.06 ± 0.11^{ab}
1.5	0.5	2.54 ± 0.30^{b}
1.5	0.05	3.82 ± 0.18^{c}
3.0	0.5	2.46 ± 0.22^{ab}
3.0	0.05	1.92 ± 0.19^{a}

* Concentration of BA and IBA in the callus induction medium (with 0.5 mgL^{-1} TDZ), as well as in the shoot regeneration medium (without TDZ). **Values represent mean ± standard error of 25 - 30 explants per treatment. Means with different letters are significantly different ($P \leq 0.05$) by Tukey's HSD test.

Table 2. Effect of adenine sulfate (Ads) and glutamine (Gln) on shoot regeneration.

Concentration (mgL^{-1})*		Total number of explants	Explants response (%)	Number of shoots/ explant**
Ads	Gln			
0	0	139	80.58	4.08 ± 0.16^{a}
5	5	142	75.35	4.54 ± 0.20^{ab}
10	10	148	74.32	4.57 ± 0.18^{ab}
15	15	128	71.09	5.44 ± 0.30^{b}
20	20	116	74.14	4.77 ± 0.18^{ab}
25	25	115	93.04	9.09 ± 0.37^{c}

*Concentration of Ads and Gln in the callus induction medium (with 1.5 mgL^{-1} BA, 0.05 mgL^{-1} IBA, and 0.5 mgL^{-1} TDZ), as well as in the shoot regeneration media (with 1.5 mgL^{-1} BA and 0.05 mgL^{-1} IBA). ** Values represent mean ± standard error of 55 - 80 explants per treatment. Means with different letters are significantly different ($P \leq 0.05$) by Tukey's HSD test.

sulphate and cytokinin on stimulating cell growth and enhancing shoot formation of *Holarrhena antidysenterica* were observed by Raha and Roy (2001). The simulative role of adenine sulfate in shoot multiplication was emphasized in different woody species such as *Melia azedarach* (Husain and Anis, 2004), *Bauhinia vahlii* (Dhar and Upreti, 1999), and *Petrocarpus marsupium* (Husain et al., 2008). Several studies showed the enhancement of shoot multiplication of jatropha in MS medium containing BA and IBA fortified by adenine sulfate and glutamine (Maharana et al., 2012; Samson et al., 2013; Mishra, 2018; Hegazi et al., 2020). The inclusion of glutamine and adenine sulfate exhibited significant effect on shoot multiplication. Among the different concentrations evaluated, 25 mgL^{-1} each of glutamine and adenine sulfate was the most effective for shoot regeneration. When comparing with the media without glutamine and adenine sulphate (4.08 ± 0.16 shoots/explant), the addition of 25 mgL^{-1} each of glutamine and adenine sulfate resulted in more than two-fold increase in the shoot number (9.09 ± 0.37 shoots/explant) (Table 2 and Figure 1e, f). The enhancement of shoot multiplication might be due to synergistic effect of glutamine and adenine sulfate. Thus, the results were similar with previous reports by Maharan et al. (2012), Samson et al. (2013), Mishra (2018) and Hegazi et al. (2020) in jaropha plant which revealed that the addition of glutamine and/or adenine sulphate significantly enhanced shoot multiplication.

Shoot elongation

The physiological role of GA_3 is well known on shoot elongation and widely used for *in vitro* regeneration of different plants (Machado et al., 2011; Gonbad et al., 2014; Padrón et al., 2020). The results showed that GA_3 at the concentration of 0.5 mgL^{-1} significantly improved shoot elongation of jatropha (2.13 ± 0.18 cm) (Table 3 and Figure 1g). The result was consistent with the results reported recently by Amiri and Mohammadi (2021) when they used the combination of BA and GA_3 for *in vitro* regeneration of Sumac plant. The established regeneration protocol of jatropha in our lab by Khemkladngoen et al. (2011) did not examine the effects of GA_3 shoot elongation. The results of this study

Table 3. Effect of GA_3 on shoot elongation in the presence of 0.5 mgL^{-1} BA.

Concentration of GA3 (mgL^{-1})	Shoot length (cm)*
0.0	1.09 ± 0.17^{a}
0.3	1.29 ± 0.17^{a}
0.5	2.13 ± 0.18^{b}
1.0	1.25 ± 0.19^{a}

*Values represent mean ± standard error of 30 – 35 explants per treatment. Means with different letters are significantly different ($P \leq 0.05$) by Tukey's HSD test.

Table 4. Effect of Gln on rooting in the presence of 0.5 mgL^{-1} IBA.

Concentration of Gln (mgL^{-1})	No. of roots/shoot	Rooted shoot (%)*
0.0	3.17 ± 1.01^{a}	40
5	1.67 ± 0.47^{a}	18
10	1.75 ± 0.39^{a}	25
15	1.75 ± 0.40^{a}	40

*Values represent mean ± standard error of 15 -20 explants per treatment. Means with different letters are significantly different ($P \leq 0.05$) by Tukey's HSD test.

indicated that GA_3 remarkably improved shoot elongation of regenerated shoots.

Rooting and acclimation

The promontory effect of IBA on *in vitro* rooting of jatropha shoots was reported previously (Deore and Johnson, 2008; Singh et al., 2010; Sharma et al., 2011). Jatropha is recalcitrant for *in vitro* regeneration at exactly rooting and acclimatization (Pankaj and Divay, 2011). Recently, Liu et al. (2015) found that the addition of glutamine to the medium in the presence of IBA effectively stimulated the initiation and growth of roots in jatropha and 16 mgL^{-1} of glutamine exhibited the best rooting rate (51.72%). The highest rooting induction efficiency (40%) was observed in medium containing 15 mgL^{-1} of glutamine, as well as that without glutamine (Table 4; Figure 1h, i). This result showed that the addition of glutamine did not significantly affect rooting efficiency, which might be due to *in vitro* elongated shoots that were regenerated in medium containing glutamine. Rooted plantlets were transplanted and acclimatized successfully to the soil for 3 weeks. The acclimatized plants exhibited normal morphological growth (Figure 1j, k).

The study described the enhanced *in vitro* regeneration protocol of jatropha from juvenile cotyledon. Explants were cultured on shoot induction medium fortified with different concentrations of adenine sulfate and glutamine. The highest number of shoots per explant with high regeneration frequency was achieved in shoot induction medium fortified by 25 mgL^{-1} each of adenine sulfate and glutamine. The results also showed the highest shoot multiplication rate in jatropha plant that has been never done before. Enhanced *in vitro* regeneration protocol would be useful for large-scale production and *Agrobacterium*-mediated transformation of elite jatropha genotypes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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