A simple and ecologically friendly method for *Jatropha curcas* tissue culture

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ABSTRACT

Some chemicals used in plant tissue culture are toxic and may cause pollution. They are not environmentally friendly and can cause damage to plant tissue, depending on the species and explant used. Therefore, this research aimed to find the most efficient and non-hazardous disinfectant used for *Jatropha curcas* leaves tissue culture. Optimum hormone balance for *J. curcas* callus culture as well as for indirect organogenesis was also tested. When 5% hydrogen peroxide (H_2O_2) was used as a disinfectant, 47.0% green explants without contamination were observed. Callus was generated with the combination of 6-benzyladenine (BA) and indole-3-butyric acid (IBA). The *J. curcas* shoots were inducted on Murashige and Skoog (MS) medium supplemented with 2 mg L^{-1} BA. These conditions provided 43.7% regeneration and an average of 20 shoots per 10 calli with an average shoot length of 17.8 mm. Roots were generated by transferring shoots to a root induction medium containing half-strength MS medium supplemented with 0.5 mg L^{-1} IBA and 100 mg L^{-1} phloroglucinol. A protocol of ecologically friendly disinfection and media for *J. curcas* tissue culture were successfully developed.

Keywords: *Jatropha curcas*, sodium hypochlorite, hydrogen peroxide, 6-benzyladenine, indole-3-butyric acid

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INTRODUCTION

Physic nut or *Jatropha curcas* L. is a drought-tolerant plant belonging to the Euphorbiaceae family. It is mostly considered to be an alternative plant source for biodiesel production. The *J. curcas* has been shown to have more significant fatty acid components when compared with alternative biodiesel plants. The seed of *J. curcas* contains high levels of unsaturated fatty acid, which comprise 44.7% oleic acid (18:1) and 32.8% linoleic acid (18:2) (Akbar *et al*., 2009). The *J. curcas*'s oils have similar characteristics to standard biodiesel. It is suitable to be used as an alternative fuel resource (Parawira, 2010). The *J. curcas* can also be used for soap production and erosion control. A methanolic extract of *J. curcas* has been found to have the ability to inhibit cancer metastasis in mice (Balaji *et al*., 2009). To improve the quality and quantity of *J. curcas*, genetic modification is one of the high potential techniques.

To create a genetically modified plant, a tissue culture method is needed for plant regeneration. Plant tissue culture consists of two main processes which are sterilization and plant regeneration. Sterilization is an essential part of the process that will improve plant regeneration, as well as achieving greater success in genetic modification (Da Silva *et al*., 2015). Many disinfectants have been used to eliminate contamination. Most plant samples are normally collected from an *ex vitro* environment with high contaminations. Mercury dichloride ($HgCl_2$) has been one of the many choices for removing bacterial and fungal contamination (Shukla *et al*.,

2013; Zhao *et al*., 2016; Yadav *et al*., 2017; Nguyen *et al*., 2018). However, it is quite toxic to both the environment and laboratory personnel. The use of less toxic chemicals for tissue sterilization would be preferred. Sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) are the ingredients in commercial bleaches. The mode of action of NaOCl is to damage the cell membrane, DNA, and protein while that of H_2O_2 is to produce hydroxyl radical to attack the lipid bilayer of the cell wall (McDonnell and Russell, 1999). These two chemicals have been used at low concentrations for sterilization in several plants.

Plantlets are regenerated and developed from calli using hormones. Auxin and cytokinin plant hormones are regularly used in plant tissue culture. Auxin is an effective hormone that is responsible for the necessary processes of cell division and cell elongation. Cytokinin is also known as a cell division regulator accountable for protein synthesis, as well as the activation of enzymes (Schaller *et al*., 2014). Thidazuron (TDZ), a cytokinin-like compound regularly used for callus and indirectly shoot induction in several plant species. However, the use of a high concentration of TDZ could inhibit shoot induction (Cao and Hammerschlag, 2000). Generally, a combination of other types of auxin and cytokinin is used to produce calli (Paranjape *et al*., 2014). Here, indole-3-butyric acid (IBA) and 6-benzyladenine (BA) were tested for the calli formation and regeneration of *J. curcas* plantlet. In this work, the effect of various disinfectants was observed to achieve optimum disinfection protocol for *J. curcas* explants. Tissue culture media with combinations of IBA and BA which are auxin and cytokinin were used to regenerate *J. curcas* plantlet.

MATERIALS AND METHODS

Optimization of Leaf Disinfectants

Young *J. curcas* leaves from the 3rd branch were collected from Suranaree University of Technology, organic farm, Nakhon Ratchasima, Thailand (Longitude 14.8726605 and Latitude 102.0250931), rinsed in tap water, and then cleaned with dishwashing detergent. The sterilization process was done using two main disinfectants which were H_2O_2 and NaOCl at 0%, 2.5%, 5%, and 10% for 5 min. Then the leaves were rinsed three times with sterile distilled water.

The sterilized leaf explants were cut into pieces approximately 0.5 × 0.5 cm^2 and incubated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) pH 5.7 supplemented with combinations of 1 mg L^{-1} benzylaminopurine (BAP) and 0.10 mg L^{-1} IBA (Subroto *et al*., 2014), with 3% sucrose, 0.7% (w/v) American bacteriological agar (Pronadisa, Laboratorios Conda, Madrid, Spain). Explants were incubated at 25°C with a light of 121 μmoL/s^2/m^2 from LED tubes. Green explants without contamination were counted after 3 weeks of culture and calculated according to equation 1. The experiments were repeated three times with five replications each.

$$\text{Green explants without contamination (\%)} = \frac{\text{Number of green explants}}{\text{Number of explants}} \times 100 \quad \text{-------(1)}$$

Media Optimization for Callus Induction

Young leaves were sterilized as mentioned above with 5% H_2O_2 (Treatment 3; Figure 1A) for 5 min. The sterilized leaves were cut into small pieces and inoculated on MS medium supplemented with combinations of BA (PhytoTechnology Laboratories, Shawnee, KS, USA) and IBA (Sigma-Aldrich, Singapore) of 0.5–2.0 mg L^{-1} (25 treatments). The medium preparation and environmental conditions for sterilization were the same as mentioned above. The percentage of callus formation was recorded after 4 weeks of culture and calculated according to equation 2. The experiments were repeated five times with five replications each.

$$\text{Callus induction (\%)} = \frac{\text{Number of callus}}{\text{Number of explants}} \times 100 \quad \text{-------- (2)}$$

Media Optimization for Shoot and Root Induction

Calli induced on MS medium supplemented with 2 mg L^{-1} BA and 1 mg L^{-1} IBA were used for shoot induction. Shoot induction was performed with combinations of BA and IBA of 0.5–2.0 mg L^{-1}. The medium preparation and environmental conditions were as mentioned above. The percentage of shoot regeneration, shoot number per callus, and shoot length (mm) were recorded after 4 weeks of culture on shoot induction medium and calculated according to equations 3, 4, and 5, respectively. The experiments were repeated five times with five replications each.

$$\text{Shoot regeneration (\%)} = \frac{\text{Number of shoots}}{\text{Number of callus}} \times 100 \quad \text{------- (3)}$$

$$\text{Shoot number per callus} = \frac{\Sigma\ \text{Number of shoots per callus}}{\text{Number of callus}} \quad \text{------- (4)}$$

$$\text{Shoot length} = \frac{\Sigma\ \text{Length of shoots}}{\text{Number of shoots}} \quad \text{------- (5)}$$

After 4 weeks, the shoots were transferred to half-strength MS medium supplemented with 100 mg L^{-1} phloroglucinol (PG) and 0.5 mg L^{-1} IBA. After one week, the rooted shoots were transferred to half-strength MS medium supplemented with 25.0 mg L^{-1} activated charcoal (AC).

Statistical Analysis

The callus and shoot induction percentage data were transformed to arcsine values using an equation fx = +ASIN [SQRT ((%Value)/100)] × (180/3.14) in Microsoft excel. Statistical analysis of the data was carried out using IBM SPSS 26 software. The experiment was set up in a completely randomized design (CRD) and the statistical analysis of data and one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests were performed at $P < 0.05$.

RESULTS AND DISCUSSION

Leaf Sterilization

Leaf disinfection was performed using NaOCl and H_2O_2. These two disinfectants showed different adverse effects on leaf explants. Leaf explants treated with NaOCl gave the highest rate of green explants without contamination (51.2%) (Figure 1A). However, all leaf explants were damaged. After 3 weeks of the tissue culture process, the leaf explants became dried and wilted. Finally, they turned brown and died (Figure 1B). The NaOCl had a stronger effect on the contaminations and the leaves explants than H_2O_2. It was able to get rid of all the contaminants, it also kills the explants. Using 2.5% H_2O_2 could not control the contamination of microorganisms. But higher than 5% H_2O_2 also killed the leaf explants similar to the use of NaOCl. Therefore, 5% H_2O_2 was the optimum concentration to use to eliminate contaminants and the leaf explants still survived (Figure 1C). It provided up to 47.0% of green explants without contamination. This condition was used in the next experiments.

In plant tissue culture, sterilization is the key to achieve the expected results. Therefore, chemicals and cleaning procedures should be considered. Simple and ecologically friendly methods were studied to find the most suitable process for *J. curcas* tissue culture via leaf explants. The results indicated that NaOCl was not suitable to sterilize *J. curcas* leaves in our experiments. However, 5% of NaOCl was successfully used to sterilize *Coccinia abyssinica* and *J. curcas* leaves (Khurana-Kaul *et al*., 2010; Guma *et al*., 2015; Liu *et al*., 2015; 2016). The performance of NaOCl tested on grapevine axillary buds showed that the contamination rate was only 19.8% when sterilized with 1.3% NaOCl for one hour (Lazo-Javalera *et al*., 2016). This discrepancy in the results might be due to the

differences in the explant, species, or different amounts of contaminations from the environments.

The H_2O_2 has rarely been used for leaf sterilization. However, using 5 and 10% H_2O_2 to sterile *Prunus perica* (L.) gave an 80% contamination rate for both shoot-tips and nodes (Al Ghasheem *et al*., 2018). In this work, H_2O_2 was used and the efficiency for leaves explant sterilization was tested. The results showed that the use of H_2O_2 was better than using NaOCl for *J. curcas* leaf sterilization.

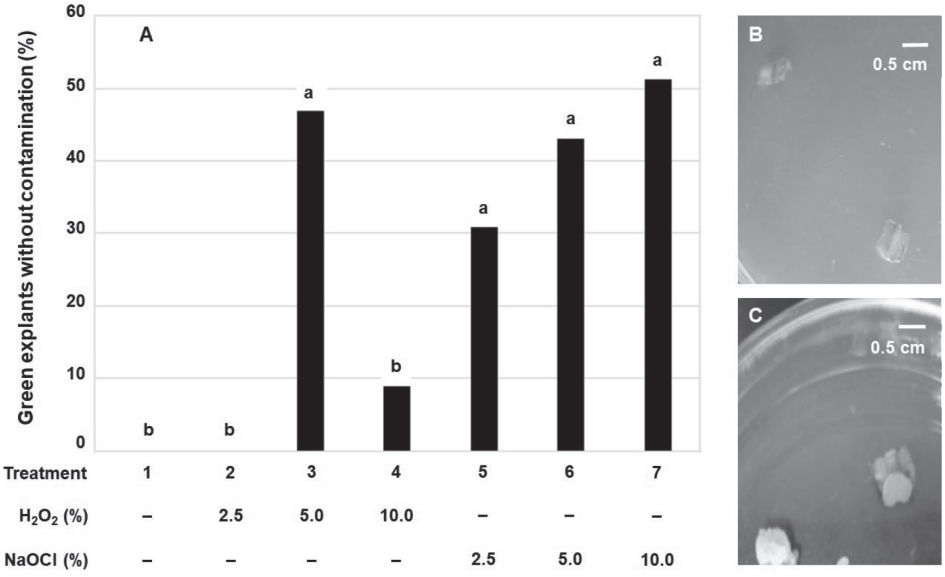

Figure 1 Disinfection of leaf explants: (A) graph shows the results of H_2O_2 and NaOCl for disinfectant optimization on leaves, (B) leaf explants sterilized with 10% NaOCl (treatment 7), (C) leaf explants sterilized with 5% H_2O_2 (treatment 3). The data was collected after 3 weeks of culture. For each treatment, different letters (a–b) represent statistically significant difference at $P < 0.05$ as determined by Duncan's multiple range tests.

Callus Formation

J. curcas callus was induced with the combination of 0.5–2.0 mg L^{-1} BA and 0.5–2.0 mg L^{-1} IBA (Figure 2). Callus could not be formed on MS medium without the two plant hormones (Figure 3A). No callus was induced with the use of a single plant hormone (Figures 3B–E). However, with the combination of plant hormones, cytokinin (BA) and auxin (IBA), 90.5% of the leaf explants expanded and calli were formed on the edges after culturing for 4–5 days (Figure 2 and Figures 3F–I). Compact calli are the best phenotype that is needed to induce shoot formation. The work of Mazumdar *et al*. (2010) has shown that after 3 weeks, *J. curcas* cotyledonary tissue can form 70.8% callus after culturing on MS medium supplemented with 1.5 mg L^{-1} BAP and 0.05 mg L^{-1} IBA. Our results showed that the combination of BA and IBA generated 90.5% callus formation from leaf explants. However, other types of cytokinin and auxin have also been used to induce *J. curcas* callus. A combination of naphthyl acetic acid (NAA) and kinetin (Kn) could induce callus from epicotyl. The MS medium supplemented with 1 mg L^{-1} NAA and 0.1 mg L^{-1} Kn gave rise to a high callus formation rate (Li *et al*., 2012). These experiments showed that the combination of cytokinin (BA) and auxin (IBA) in MS medium at various concentrations gave

a similar callus formation rate which might be due to the sensitivity of BA and IBA on *J. curcas* callus formation might not be high. However, regarding the morphology of the callus, MS medium supplemented with 2 mg/L BA and 1 mg/L IBA was the optimum medium to induce nice compact *J. curcas* calli for shoot regeneration.

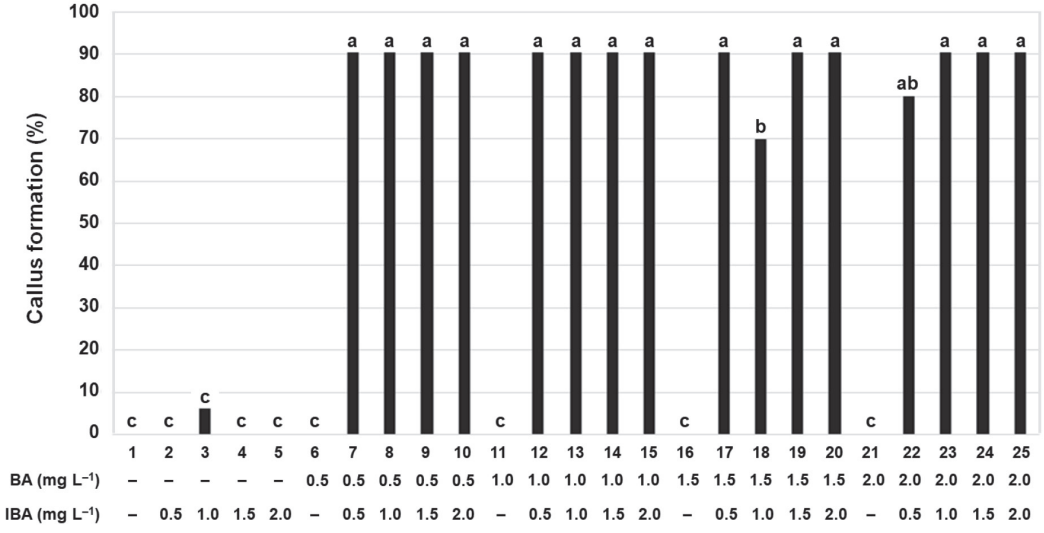

Figure 2 Effect of the combination of 6-benzyladenine (BA) and indole-3-butyric acid (IBA) on callus formation. Explants were cultured on media with 25 different combinations of BA and IBA plant hormones. The data was recorded after 4 weeks of culture. For each treatment, different letters (a–c) represent statistically significant difference at $P < 0.05$ as determined by Duncan's multiple range tests.

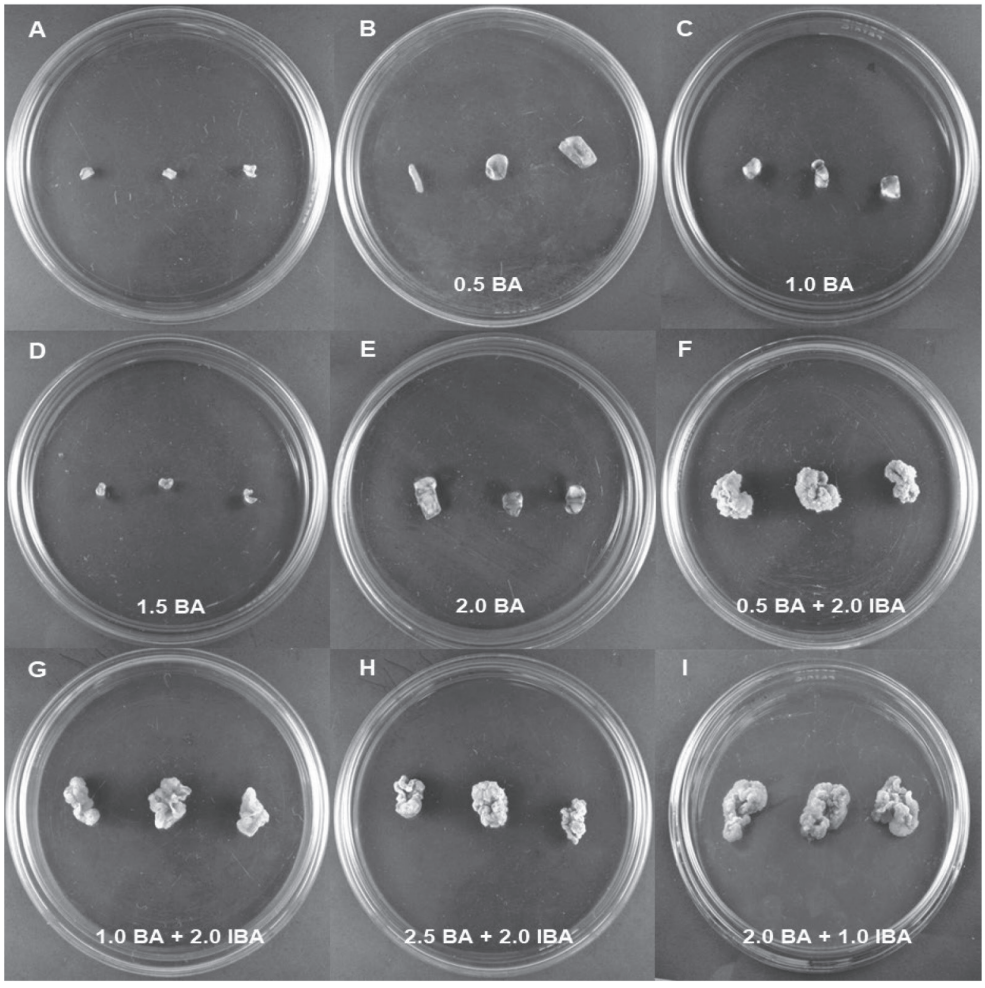

Figure 3 Effect of the combination of 6-benzyladenine (BA) and indole-3-butyric acid (IBA) on callus induction: (A) induced calli on Murashige and Skoog (MS) medium without plant hormone, (B–E) induced calli on MS medium supplemented with only BA, (F–I) induced calli on MS medium supplemented with a combination of BA and IBA. The values are in mg L^{-1}.

Shoot and Root Induction

After 4–6 weeks, *J. curcas* calli were induced with the combination of BA and IBA to produce the shoot. Supplementation with only BA at 2.0 mg L^{-1} gave rise to 43.7% of shoot regeneration with 20 shoots per 10 calli. The longest shoot length was 22.1 mm with an average of 17.8 mm per shoot (Table 1 and Figure 4A).

For root induction, the shoots were transferred to half-strength MS medium supplemented with 0.5 mg L^{-1} IBA and 100 mg L^{-1} PG (Figure 4B). After 7 days, long white roots were observed from the callus connected with the shoot. Then, the rooted shoots were transferred to a root elongation medium containing a half-strength MS medium supplemented with 25.0 mg L^{-1} AC to elongate the root (Figures 4C–D). In the root elongation medium, the productions of branch roots and root hairs were observed.

Mweu *et al*. (2016) showed that shoots induced from *J. curcas* callus from the greenhouse-grown plants on MS medium supplemented with

4 plant hormones, i.e., 1.5 mg L^{-1} BAP, 0.6 mg L^{-1} Kn, 0.3 mg L^{-1} indole-3-acetic acid (IAA), and 0.1 mg L^{-1} TDZ, after 8 weeks was up to 8.25 cm. Moreover, Subroto (2014) observed 45.8% shoot induction on MS medium supplemented with 2 mg L^{-1} BAP, 0.05 mg L^{-1} IBA, and 0.5 mg L^{-1} gibberellin (GA_3). Since IAA is a heat-sensitive hormone and TDZ has been shown to be somewhat toxic, these two hormones are not simple to prepare and use. An alternative way of direct shoot organogenesis induction from juvenile cotyledon *J. curcas* was achieved with MS medium supplemented with 0.5 mg L^{-1} BAP, 0.2 mg L^{-1} IBA, and 1.0 mg L^{-1} TDZ. Shoots were induced up to 3 shoots per explant within 6 weeks (Tsegay *et al*., 2017). However, TDZ is known to be a herbicide that is toxic to fish and aquatic organisms and it is not easily degraded. It also irritates the eye, skin, and mucous membranes in humans (Paranjape *et al*., 2014). Additionally, herbicide 2,4-Dichlorophenoxyacetic acid (2,4-D) is a toxic herbicide plant hormone that is not easily degraded. Contamination of 2,4-D has been found in laboratories and environments (Dos Santos Costa *et al*., 2020). In our work only BA and IBA were used and able to induce shoot from compact *J. curcas* calli. Both single hormones or the combination of the two hormones can give rise to *J. curcas* shoot within 4 weeks.

Table 1 Effect of the combination of 6-benzyladenine (BA) and indole-3-butyric acid (IBA) on shoot induction

Medium	BA (mg L^{-1})	IBA (mg L^{-1})	Shoot regeneration (%)	Shoot number per 10 calli	Shoot length (mm)
1	0.00	0.00	0.00^{b}	0.00^{b}	0.00^{c}
2	0.00	0.50	0.00^{b}	0.00^{b}	0.00^{c}
3	0.00	1.00	0.00^{b}	0.00^{b}	0.00^{c}
4	0.00	1.50	0.00^{b}	0.00^{b}	0.00^{c}
5	0.00	2.00	0.00^{b}	0.00^{b}	0.00^{c}
6	0.50	0.00	7.06 ± 15.78^{b}	4.00 ± 0.89^{b}	3.41 ± 7.63^{bc}
7	0.50	0.50	7.06 ± 15.78^{b}	2.00 ± 0.45^{b}	3.39 ± 7.57^{bc}
8	0.50	1.00	7.06 ± 15.78^{b}	2.00 ± 0.45^{b}	3.05 ± 6.83^{bc}
9	0.50	1.50	21.17 ± 19.32^{ab}	6.00 ± 0.55^{b}	10.22 ± 9.54^{b}
10	0.50	2.00	0.00^{b}	0.00^{b}	0.00^{c}
11	1.00	0.00	10.95 ± 24.491^{b}	4.00 ± 0.89^{b}	2.01 ± 4.50^{bc}
12	1.00	0.50	14.11 ± 19.32^{b}	4.00 ± 0.55^{b}	7.01 ± 10.02^{bc}
13	1.00	1.00	7.06 ± 15.78^{b}	2.00 ± 0.45^{b}	2.74 ± 6.12^{bc}
14	1.00	1.50	18.00 ± 25.601^{b}	6.00 ± 0.89^{bc}	2.67 ± 3.98^{bc}
15	1.00	2.00	10.95 ± 24.49^{b}	4.00 ± 0.89^{b}	7.13 ± 11.21^{bc}
16	1.50	0.00	26.17 ± 19.32^{ab}	8.00 ± 0.84^{b}	5.41 ± 4.98^{bc}
17	1.50	0.50	8.68 ± 15.28^{b}	2.00 ± 0.45^{b}	1.72 ± 3.85^{bc}
18	1.50	1.00	1.63 ± 3.64^{b}	0.00^{b}	0.00^{c}
19	1.50	1.50	14.11 ± 19.32^{b}	10.00 ± 1.41^{b}	5.19 ± 7.12^{bc}
20	1.50	2.00	10.95 ± 24.49^{b}	6.00 ± 1.34^{b}	1.55 ± 3.47^{bc}
21	2.00	0.00	43.70 ± 10.67^{a}	20.00 ± 1.23^{a}	17.80 ± 5.80^{a}
22	2.00	0.50	10.95 ± 24.49^{b}	6.00 ± 1.34^{b}	1.67 ± 3.73^{bc}
23	2.00	1.00	25.06 ± 24.22^{ab}	6.00 ± 0.55^{b}	7.76 ± 9.32^{bc}
24	2.00	1.50	14.11 ± 19.32^{b}	4.00 ± 0.55^{b}	4.31 ± 6.11^{bc}
25	2.00	2.00	0.00^{b}	0.00^{b}	0.00^{c}

Note: Data in the same column followed by different letters are significantly different at $P < 0.05$ as determined by Duncan's multiple range tests. Shoot regeneration, shoot number per callus, and shoot length were recorded after 4 weeks of culture.

Similarly, 52.0% *J. curcas* root could be induced within 2–3 weeks with MS basal medium supplemented with 0.2 mg L^{-1} IBA with 5.6 roots per shoot, and 8.7 cm of root length (Datta *et al*., 2007). The IBA, which is an auxin, plays a critical role in root initiation. Moreover, the balancing of auxin and cytokinin is important for root induction. The highest efficiency of root induction was presented by using a half-strength woody plant medium (WPM) supplemented with 0.5 mg L^{-1} IBA and 30.0 mg L^{-1} PG. Root formation was up to 83.0%, which could generate root up to 3.1 roots per shoot within 6 weeks (Daud *et al*., 2013). In our study, roots were also generated from the modification of the above root induction medium. A few plantlets were obtained. The strong plantlets were later acclimatized and transferred to soil.

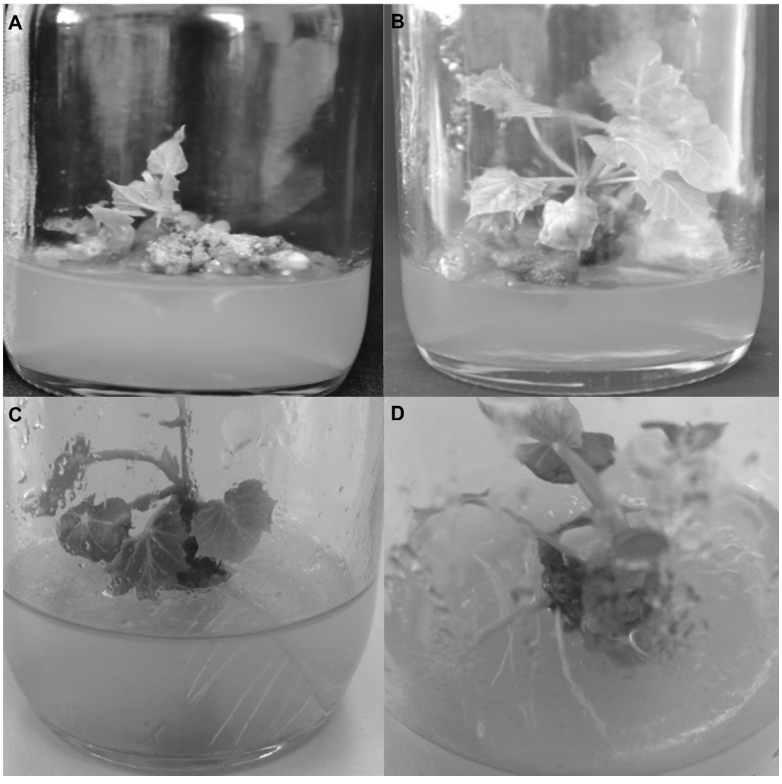

Figure 4 Regeneration of shoot and rooted shoots: (A) regenerated shoot on medium supplemented with 2 mg L^{-1} BA (6-benzyladenine), (B) elongated shoot on Murashige and Skoog medium supplemented with 0.5 mg L^{-1} IBA (indole-3-butyric acid) and 100 mg L^{-1} PG (phloroglucinol), (C–D) induced root on half-strength Murashige and Skoog medium supplemented with 25 mg L^{-1} AC (activated charcoal)

CONCLUSIONS

Sterilization of leaves for plant tissue culture with non-hazard sanitizer can be done. The H_2O_2 of 0.5% was the optimum concentration for *ex vitro J. curcas* leaf explants sterilization. *J. curcas* calli were produced from leaves on MS medium with the combination of BA and IBA. Indirect shoots were developed on MS medium supplemented with only BA. Then, the root was induced on a half-strength MS medium supplemented with IBA and PG. The roots were able to elongate and branch on a half-strength MS medium supplemented with AC. The plantlets were transferred to the soil.

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