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Research Article
Regeneration of *Jatropha curcas* Using Cotyledonary Petiole Treated with Silver Nitrate Generated from Both *in vitro* and *in vivo* Planting

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Abstract

Background and Objective: The possibilities of using *J. curcas* seeds oil as bio-fuel in Nigeria requires large scale production of the plant which can be achieved through plant tissue culture technique. This study aimed to determine the effects of varying concentrations of silver nitrate ($AgNO_3$) solution on the regeneration of *J. curcas* from cotyledonary petiole explants. **Materials and Methods:** The experimental layout followed completely randomized design with 20 replications for factors such as varying concentrations of silver nitrate, soaking period and orientation of the explants on MS-media. Data were analyzed using analysis of variance and means were separated using Duncan Multiple Range Test at $p<0.05$. **Results:** Application of $AgNO_3$ solution at 10 mg L^{-1} promoted greater percentage shoot buds regeneration and number of shoot buds per explant. Twenty minutes process-time favoured higher shoot buds regeneration and placement of explants in slant position was found to be conducive orientation for regeneration of shoot buds. L-arginine between 7 and 10 mg L^{-1} promoted elongation of regenerated shoot buds. Rooting of the elongated shoot buds was faster in culture receiving 20 mg L^{-1} of indole-3-butyric acid (IBA). Regeneration of shoot buds regeneration was better in *in vitro* than in *in vivo.* It took 126-135 to achieve regeneration of plantlets. **Conclusion:** Recovery of plantlets was better when *in vitro* cotyledonary petiole explants were treated with 10 mg L^{-1} of $AgNO_3$ solution, soaked for 20 min and placed in a slanted position on MS media and receiving further treatments of 7-10 mg L^{-1} of L-arginine and 20 mg L^{-1} of IBA.

Key words: Cotyledonary petiole, indole-3-butyric acid, L-arginine, regeneration, silver nitrate, tissue culture technique, shoot buds

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Jatropha curcas, is a large drought-resistant multipurpose shrub with several potentials. Special interest has been shown in the cultivation of *J. curcas* plantation because of its seed oil, which could be easily converted into quality bio-diesel[1]. More recently, the clear oil expressed from the seed has been suggested for energetic purposes as a substitute for diesel during[2] the World War II. The usefulness of this plant most importantly its seed calls for the need to enter into massive production through the technique of tissue culture.

Plant tissue cultures are known to be used to create a quick and efficient way to regenerate many plants from a single piece of a "mother" plant. An explant is a piece of living tissue that is transferred to an artificial growth medium. The explant could come from numerous parts of the plant, including the leaf, stem, root, flower or seed[3]. There have been many studies using various tissue culture methods on different explants of *J. curcas*[4-6]. Workers found out good callusing on media with different combinations of benzene amino purine (BAP) and indolebutyric acid (IBA) (90%) from 3rd position leaves, leading to a protocol for successful indirect regeneration of *J. curcas*[7]. They also reported direct adventitious shoot regeneration of *J. curcas* and used hypocotyl, leaf and petiole explants. In 2005, auxiliary nodes were used for shoot bud proliferation in addition to leaf explants for adventitious shoot regeneration[8]. A successful protocol for somatic embryogenesis using seven leaves from seven months old *J. curcas* plants was reported[9] in 2007. A year later, indirect regeneration protocol from *J. curcas* leaf explants was reported by Li *et al.*[10]. About the same time, 80% rooting using full strength MS medium with 0.5 mg L^{-1} IBA was recorded by a researcher and he developed a protocol for direct shoot regeneration from leaf explants[10]. Recently, direct regeneration from leaf, petiole and cotyledonary petiole explants has been recorded with established protocol[11,12].

J. curcas, most importantly its seed has great economic, agricultural and nutritional potential[13]. In recent times, the plant has attracted attention of various research organizations, governments, public and international developmental agencies and industries in the tropics and subtropics due to its adaptability to semi-arid marginal sites, the possibility of using its oil as a diesel fuel substitute and its role in erosion control[13]. Massive production of this plant that will promote its plantation within short period of time via the technique of tissue culture has not been given serious consideration most importantly in Nigeria which the current study aimed to address.

MATERIALS AND METHODS

Study site and sample collection: The research was carried out at the National Centre for Genetic Resource and Biotechnology (NACGRAB), Ibadan, Oyo state, Nigeria between October, 2017 and March, 2018. The materials used were supplied by the Tissue Culture Unit of the centre. The reagents used were of analytical grade. Mature seeds of *J. curcas* were collected from Faculty of Agricultural Unit of University of Ilorin, Ilorin, Nigeria.

Sterilization of glass wares: Glass wares after being thoroughly washed using liquid soap and hypochlorite for an hour were subjected to both dry and wet sterilization in an oven an autoclave, respectively.

Preparation of Murashige and Skoog media (MS): Prepared MS media contained all the growth adjuvants such as macro and micro nutrient, carbon source and vitamins. The pH of the MS medium was adjusted to 5.8 with 0.1 N NaOH and 0.1 N HCl before adding gelling agent of 2 g phytagel (Sigma). The media was then covered with a foil paper and heated until a clear solution was seen. The molten media was distributed into culture bottles and subsequently autoclaved before inoculation.

Disinfection of seeds: Shelled and unshelled seeds of the plant (Fig. 1) were surface sterilized with liquid soap and properly rinsed with several distilled water. It should be noted that disinfections of the seeds were done using absolute sodium hypochlorite before inoculation.

Fig. 1(a-b): *Jatropha curcas* seeds (a) With shells and (b) Without shells

Inoculation of growth media with *J. curcas* seeds: This was done in laminar flow hood using atomizer to make the chamber sterile. Forceps and blade holder were again sterilized under the spirit lamp to begin the inoculation process. Shelled seeds and unshelled seeds were inoculated on the growth media and germinations were monitored to generate *in vitro* cotyledonary petiole explants. While *J. curcas* seeds were planted in potted soil in screen house to generate *in vivo* cotyledonary petioles explants.

Regeneration of shoot buds from cotyledonary petiole as influenced by silver nitrate: Twenty-day old cotyledonary petiole explants each of 0.5 cm length grown from *in vivo* and *in vitro* propagation were treated with different concentrations of $AgNO_3$ solution (0, 10, 20, 30, 40 mg L^{-1}). The explants were soaked at different time periods (0, 10, 20, 30 and 40 min) in each of the concentrations of $AgNO_3$ solution. The cotyledonary petioles explants generated from *in vivo* and *in vitro* propagation were placed in different orientations: horizontal, vertical and slant on hormone-free MS medium for regeneration of explant. The percentage induction of shoot buds and the number of shoot buds per explants was determined after 30 days of culture.

Shoot buds elongation culture: Regenerated shoot buds along with the mother tissues was moved to *Jatropha* shoot elongation media (JSR) after the formation of calli. JRS contains full strength MS and some other growth adjuvants. Various concentration of L-arginine (0, 5, 7, 10 and 20 mg L^{-1}) was supplemented with the elongation medium and the length of the elongated buds recorded after 15 days of culture.

Rooting culture: Shoots of 10 mm in length was isolated from the mother tissue and transferred to half-strength MS medium containing indole-3-butyric acid (IBA, Sigma-Aldrich Co., St Louis, MO, USA of different concentrations (0,0.5, 0.1, 1.5 and 3.0 mg L^{-1}) and number roots recorded after 40 days in culture media.

Acclimatization and transplantation of the regenerated plantlets: The regenerated plantlets were taken from culture bottles, washed properly and gently for purpose of removing residuals of medium and thereafter transplanted to a tray containing sterile polythene bags filled with sterile soil. The tray was covered with a transparent plastic sheet for 2 weeks. The established plants were then transferred to a screen house for acclimatization and further growth.

Statistical analysis: All the experiments were set up in a completely randomized factorial design with 20 replicates per treatment. Data were analyzed used Statistical Package for Social Science (SPSS) 20.0 software and means were separated by Duncan's multiple range test[14] at $p<0.05$. Figures were used where appropriate.

RESULTS

In this study, the overall Univariate analysis variance of *in vivo* and *in vitro* cotyledonary petiole explants that were treated with different concentration of silver nitrate ($AgNO_3$) soaked at different time and placed in MS medium at different orientation on induction of shoot bud is presented in Table 1. All the factors considered were found to be significant $p<0.05$. The interaction effects were also significant. The significant interactions of all the factors required that the main effect had to be down played and that the induction of the shoot buds depends largely on the factors that were considered (Table 1).

Silver nitrate effect on regeneration percentage and number of shoot buds per explant of *J. curcas*: Table 2 shows the effect of different concentration of $AgNO_3$ on the regeneration percentage and number of shoot buds per explant. In both *in vitro* and *in vivo* as sources of cotyledonary petiole explants, varying concentration $AgNO_3$ showed significantly higher shoot buds regeneration compared with control (Table 2). In *in vitro*, the concentration of $AgNO_3$ at 10 mg L^{-1} was found to be most effective with highest percentage of shoot buds induction (76.80%) and followed by 20 (39.40%) and 30 (28.81%) and 40 mg L^{-1} (22.35%). Significantly lowest shoot buds regeneration was recorded from the control with percentage value of 4.00 % (Table 2). Whereas *in vivo*, significantly higher percentage shoot buds regeneration was recorded for 20 mg L^{-1} when compared to other cotyledonary petiole explants. Similar trends of results were recorded for regeneration percentage (Table 2). It should be noted that in both *in vitro* and *in vivo*, higher concentration (30-40 mg L^{-1}) of $AgNO_3$ reduced the number of shoot buds regenerated compared to lower concentrations (10-20 mg L^{-1}) (Table 2). Similarly, shoot bud regeneration was greater *in vitro* than *in vivo* cotyledonary petiole explants (Table 2).

Effect of duration on the regeneration percentage and number of shoot buds per explants of *J. curcas*: The results of *in vitro* cotyledonary petiole explants showed that regeneration of shoot buds and number of buds per explants

Table 1: Univariate factorial results of the regenerated shoot buds

Sources	Type III sum of squares	df	Mean square	F	Significant
Source of explant	43.740	1	43.740	83.004	0.000
Error within	1.060	4	0.265	0.503	0.734
Concentration	76.643	4	19.161	36.361	0.000
Error within	18.340	16	1.146	2.175	0.005
Orientation	45.523	2	22.762	43.194	0.000
Error within	3.093	8	0.387	0.734	0.662
Duration	30.513	2	10.171	19.301	0.000
Orientation vs duration	13.877	6	2.313	4.389	0.000
Concentration vs duration	17.903	12	1.492	2.831	0.001
Concentration vs orientation	21.227	8	2.653	5.035	0.000
Source vs concentration vs orientation vs duration	92.567	82	1.115	2.116	0.000
Error	236.080	448	0.527		
Total	758.000	600			
Corrected total	609.993	599			

vs: Interaction

Table 2: Silver nitrate effects on regeneration (%) and number of shoot buds/explants of *J. curcas* after 30 days

Source of explants	Concentration of $AgNO_3$ (mg L^{-1})	Regeneration (%)	Number of shoot buds/explants
In vitro	0	4.00±0.32^{e}	1.16±0.41^{a}
	10	76.80±1.23^{a}	10.66±1.59^{c}
	20	39.40±0.65^{c}	5.33±0.83^{b}
	30	28.81±0.58^{b}	4.00±0.75^{a}
	40	22.35±0.58^{d}	3.00±0.64^{b}
Mean		34.27±0.67	4.83±0.84
p-value		0.01	0.02
In vivo	0	0.55±0.12^{c}	0.16±0.16^{c}
	10	6.90±0.48^{a}	2.00±0.61^{a}
	20	9.18±0.48^{a}	2.66±0.62^{a}
	30	5.73±0.37^{b}	1.66±0.48^{b}
	40	3.45±0.30^{b}	1.00±0.39^{b}
Mean		5.16±0.35	1.49±0.45
p-value		0.03	0.02

Data in the same column followed by different letters are significantly different at p<0.05

Table 3: Effect of duration on the regeneration (%) and number of shoot buds/explant of *J. curcas* after 30 days

Source of explants	Duration (min)	Regeneration (%)	Number of shoot buds/explants
In vitro	0	3.33±0.33	1.16±0.66
	10	42.57±0.56^{n}	3.86±0.65^{a}
	20	75.13±1.09^{a}	10.56±1.56^{b}
	30	74.64±0.81^{a}	9.53±0.93^{b}
	40	28.83±0.45^{c}	2.26±0.52^{a}
Mean		44.90±0.65	5.47±0.80
p-value		0.03	0.04
In vivo	0	0.33±0.00	1.00±0.00
	10	6.04±0.36^{b}	1.60±0.42^{b}
	20	10.57±0.50^{a}	2.80±0.58^{a}
	30	5.51±0.39^{b}	1.46±0.45^{b}
	40	5.02±0.11^{c}	1.33±0.13^{c}
Mean		5.49±0.27	1.64±0.32
p-value		0.02	0.03

Data in the same column followed by different letters are significantly different at p<0.05

was significantly influenced by process-time (Table 3). Twenty minutes process-time resulted in greater percentage of shoot buds regeneration (75.13%) and number of shoot buds per explant than other periods. (Table 3). Significantly lowest percentage regeneration and number of shoot buds/explants were recorded from the control (Table 3). The same trend of results was recorded for *in vivo* cotyledonary petiole explants. Like the effect of concentrations, the means shoot buds regeneration as well as number of shoot buds per explant for process-time was higher in *in vitro* than *in vivo* (Table 3).

Table 4: Effect of orientation on the regeneration (%) and number of shoot buds/explants of *J. curcas* after 30 days

Source of explants	Orientation	Regeneration (%)	Number of shoot buds/explants
In vitro	Horizontal	67.42±0.41[b]	5.40±0.52[b]
	Slant	79.10±0.37[a]	10.66±1.59[c]
	Vertical	28.80±0.35[c]	4.00±0.75[a]
Mean		58.44±0.38	6.69±0.76
p-value		<0.001	0.03
In vivo	Horizontal	10.00±0.79[a]	1.90±0.41[a]
	Slant	7.37±0.96[b]	1.40±0.37[b]
	Vertical	6.32±0.52[b]	1.20±0.35[b]
Mean		7.89±0.76	1.50±0.37
p-value		0.03	0.02

Data in the same column followed by different letters are significantly different at p<0.05

Table 5: Effect of L-arginine on the elongation of the regenerated shoot buds from *in vitro* explants of *J. curcas* after 15 days

Concentration of L-arginine (mg L^{-1})	Length of shoot buds (cm)
0	0.20±0.03[b]
5	0.47±0.06[a]
7	0.50±0.05[a]
10	0.50±0.05[a]
20	0.33±0.00[b]
Mean	0.15±0.04
p-value	0.03

Data in the same column followed by different letters are significantly different at p<0.05

Effect of orientation on the regeneration percentage and number of shoot buds per explants of *J. curcas*: Considering the effect of orientation that is the horizontal, slant and vertical placement of explants on the MS medium, significant differences were recorded in both *in vitro* and *in vivo* regeneration of cotyledonary petiole explants. (Table 4). *In vitro*, slant placement showed significantly highest regeneration of shoot buds of 79.10±0.37% and number of shoot buds per explant (10.66±1.56) and followed in decreasing order of magnitude by those of horizontal and vertical placement (Table 4). Whereas, *in vivo* source of explants, horizontal placement markedly favored higher shoot buds regeneration percentage and number of buds per explant (Table 4). This was closely followed by slant. Significantly lowest shoot buds regeneration percentage and number of buds per explant was recorded from vertical orientation of cotyledonary petiole explants (Table 4).

In Fig. 2a, the *in vitro* explants treated with 10 mg L^{-1} of silver nitrate, soaked for 20 min and placed in slant position showed robust shoot buds regeneration when compared to those of *in vivo* explants receiving 20 mg L^{-1} of silver nitrate, 20 min soaking period with horizontal placement (Fig. 2b).

Elongation of the regenerated shoot buds from *in vitro* explant of *J. curcas* as affected by L-arginine: The elongation of regenerated shoot buds as influenced by varying concentration of L-arginine is shown in Table 5. The most suitable concentration for promoting the elongation of shoot was between 7 and 10 mg L^{-1}. The effect of these concentrations was statistically similar. It was however, significantly higher than those recorded for 5 and 20 mg L^{-1} and the control. Shoot buds receiving the most effective treatments showed good vigor and healthy shoot buds (Table 5) after 15 days of culture. When L-arginine was used at a higher concentration (20 mg L^{-1}) and concentration as low as 5 mg L^{-1} the elongation of shoot buds was markedly inhibited (Table 5).

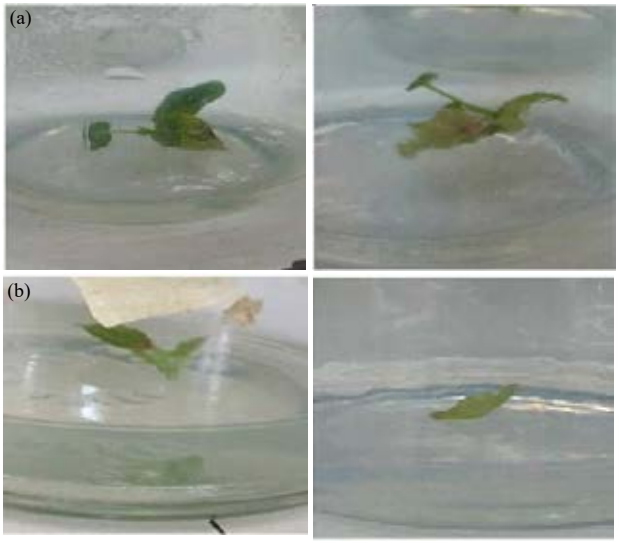

Fig. 2(a-b): Direct induction of adventitious buds from cotyledonary petiole explants of *J. curcas*, (a) Treatment of *in vitro* (with slant orientation) and (b) *In vivo* (horizontal orientation) petiole explants for 20 min duration $AgNO_3$ treatment

Effect of indole-butyric acid (IBA) on the rooting of regenerated shoots from explant of *J. Curcas*: The results obtained at day 40 after treatment of elongated *J. curcas* with indole-butyric acid (IBA) at varying concentration for root

Fig. 3: Acclimatization of regenerated plantlets

Table 6: Effects of IBA on the rooting of regenerated shoots from explant of *J. Curcas*

Concentration of indole-butyric acid (IBA) (mg L^{-1})	Number of roots/shoot
0	0.00 ± 0.03^{b}
5	0.00 ± 0.06^{a}
7	1.33 ± 0.05^{a}
10	2.33 ± 0.05^{a}
20	3.67 ± 0.00^{b}
Mean	1.47 ± 0.04
p-value	<0.001

Data in the same column followed by different letters are significantly different at $p<0.05$

initiation is presented in Table 6. It tooks a longer time for rooting to be initiated. However, number of roots per shoot increased with increase in concentration of IBA. It showed that the response of the shoot to this hormone was dose dependent (Table 6). Root initiation was not possible when no IBA was used (control) as well as for those receiving 5 mg L^{-1} of IBA (Table 6). The resulting plantlets were transferred to the screen house for acclimatization as shown in four weeks after rooting (Fig. 3) It should be noted that in this study, it tooks 126-135 days to obtain regenerated plantlets.

DISCUSSION

J. curcas is a multipurpose, drought resistant, perennial plant. The plant is currently gaining a lot of attention being a good source of bio-fuel. In this case, attention should therefore be shifted to develop a relatively efficient protocol for regeneration of plantlets with minimal contamination via the technique of plant tissue culture. In this study, the effect of varying concentration of silver nitrate were tried on the regeneration of *J. curcas* plantlets and the results showed that several factors such concentration of pre-treated cotyledonary petiole explants with silver nitrate, duration of process-time, orientation of the explant on the MS media and source of explants most importantly *in vitro* propagation should be taken into consideration in order to regenerate shoot buds development. It had been earlier reported[15] that factors such as growth regulators, orientation of the explant on the media have great effect on the response of the explants. It is worth of mentioning that, application of silver nitrate solution at 10 mg L^{-1} is considered optimum for promoting the regeneration of shoot buds in those cotyledonary petiole explants derived from *in vitro* source. The results were not in line with the findings of Liu *et al.*[16] where growth media supplemented with 20 mg L^{-1} of Thidiazuron (TDZ) significantly increased regeneration percentage and number of shoot buds per plant when *in vitro* cotyledonary petiole explants were used. The variation could be ascribed to growth adjuvant used. Also, the present investigation differed slightly as to the number of days used to regenerate the plantlets which took the authors 105 days as against the 135 days recorded in this study. Other workers have reported[7,18,15]. better regeneration of adventitious shoot in *in vitro* multiplication of *Jatropha curcas*.

With respect to process-time, cotyledonary petioles explant soaked for 20 min in silver nitrate solution gave the best result in terms regeneration of shoot buds and number of shoot buds/explant. All other durations markedly inhibited shoot bud regeneration but were better than the control. The present results indicated that silver nitrate as ethylene modulator is only required for short period of time during induction of shoot buds formation. The results are closely consistent with previous studies in *J. curcas* and glycine max[16,17].

In all the orientations investigated, the present study revealed that slant placement of the explants on the media was confirmed to be much more conducive orientation for regeneration of shoot buds. The percentage of induction of shoot buds and the number of induced shoot buds recorded in this study differed significantly under different orientations and the source of explants and this was at variance with observation reported in earlier researches by Kondamudi *et al.*[15] and Liu *et al.*[16]. Available information have established that poor quality of the regenerated shoot buds has been a major problem that hinders further development

of the regenerated buds in *J. curcas*[15]. In the present study, the problem was partially taken care of by using low concentration of silver nitrate and short treatment duration. Visual observation showed that there was an improvement of the quality of the regenerated shoot buds and shoot bud elongation was further enhanced when the explants were exposed to 5-7.5 mg L^{-1} of L-arginine L-arginine an amino acid that was scarcely used in plant tissue cultures and it has been reported to have beneficial effects in tissue culture *J. curcas* and apple[18,19]. In this study, concentration as low as 5 mg L^{-1} and as high as 7.5 mg L^{-1} are considered optimum for the elongation of shoot buds. Like GA_3, L-arginine may have positive effect on facilitating the elongation of regenerated buds[6,18].

It has been established that for the purpose of obtaining intact plants, auxin, is frequently supplemented into the rooting medium[19]. The rooting of shoot could indeed begin starting from 20 days of culture and because of slow root development, there was need to extend the time to 40 days for purpose of obtaining satisfying rooting efficiency. The result is suggestive of the fact that 40 days was suitable for rooting. Optimum concentration to achieve better root development was the highest concentration of the IBA (20 mg L^{-1}). The present study slightly agreed to the report of Liu *et al.*[16], who reported 30 days for rooting efficiency in *J. curcas* treated with TDZ. The variation in number of days for rooting efficiency could be attributed to the growth adjuvant used. This implies that the response of cotyledonary petiole explants of *J. curcas* will vary with different growth adjuvant as pre-treatment chemicals. Finally, regenerated plantlets were successfully acclimatized in potted soil in the screen house and no abnormal development were observed up to this point.

CONCLUSION

This study has shown that successful regeneration of shoot buds was best achieved if *in vitro* cotyledonary petiole explants that were treated with 10 mg L^{-1} silver nitrate solution, soaked for 20 min and placed in slant position. Further still, shoot bud elongation and rooting for regeneration of plantlets were favored upon applications of L-arginine and IBA at 7-10 mg L^{-1} and 20 mg L^{-1} respectively.

SIGNIFICANCE STATEMENT

This study discovers that using silver nitrate at 10 mg L^{-1} as growth adjuvant is beneficial for large production of *J. curcas* plantlets most importantly when the cotyledonary explants were soaked for 20 min and placed in slant orientation on MS-media. This study will help researcher to uncover the critical role of silver nitrate when used optimally in growth modulation of *J. curcas* that many other researchers were not able to explore. Thus, a new theory on this compound when utilized appropriately or in combination with other concentrations of compounds such as L-arginine and IBA may be arrived at.

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