Utilizing Plant Tissue Culture Protocol for Propagation of *Jatropha curcas*-A Semi-Arid Tree Species in Maiduguri, Nigeria

Abstract

In the present study an attempt has been made to develop a suitable protocol for efficient micropropagation from shoot tip explants excised from *Jatrophacurcas*tree growing within the north East Zonal Biotechnology Center, University of Maiduguri Borno State Nigeria in 2016. The effects of different media concentration on the species growth and development were assessed. The explant were cultured at different level of plant growth regulators Benzyle adenine (BA) and Indole-3-buteric acid (IBA) in Murashige and Skoog (MS) full strength basal media for shoot induction and multiplication. In vitro shoot multiplications were obtained successfully from all shoot tips explants after 30 days of culture. The parameters recorded were statistically analyzed and means were compared using Duncan's Multiple Range Test ($P<0.05$). Best response was observed in a MS medium (full Strength) supplemented with low level of BA (0.5mg/l) and IBA (0.2mg/l) showing shoot height of 1.567cm. The minimum percentage of response was obtained in media combination containing BA 1.0mg/l and 0.4mg/l IBA. Data obtained also showed a significant ($P<0.05$) difference in the mean number of leaves, axillary shoot and the shoot length induced by the explant. The study reveals that high phenolic exuded by explant leading to hyperhydrocity, and low moisture condition hinders explant growth and development. None of the explant rooted when cultured on the rooting media (half MS media supplemented with Plant Growth Regulators IBA and NAA) but rather produces some substance appearing to be callus at the base of the plantlets which forced for histology. The histological studies revealed that, no any irregularities in the tissues structures and organization that might prevent the plantlet from rooting. From the result of this study, it can be concluded that the use of full-strength MS media supplemented with a very low Cytokinin and Auxin could be suitable for propagating *Jatropha curcas* in vitro. It is recommended that further research be undertaken to improve

proliferation and rooting efficiency, for possibilities of substituting the conventional method of propagating of these tree species.

Key words
Invitro Propagation, *Jatropha curcas,* Plant Growth Regulators, Shoot Induction, Histology, MS medium.

1.0 Introduction

Tree species play a vital role in supporting the biodiversity of plant and animal life within arid regions and ecosystems at large. Biodiversity serves as a reservoir of species richness and provides a crucial buffer against potentially harmful environmental changes and economic fluctuations. Notably, plant genetic resources form the foundation of global food security, sustaining livelihoods by offering essential products such as fuel, food, timber, herbs, and fodder(Aerts andHonnay, 2011).

Additionally, trees act as vital wildlife habitats, while also fulfilling crucial functions like soil nutrient fixation, providing shelter, and contributing to air and water quality. *Jatropha curcas*, for instance, is extensively cultivated as a living fence to protect crops from livestock in rural areas, with various parts of the plant having medicinal value. *Jatropha curcas,* has garnered considerable attention due to its potential as a viable biofuel source (Devappa *et al.,* 2010). Theyexhibit a remarkable ability to withstand drought conditions as it demonstrates robust growth on degraded soils, necessitating only minimal fertilizer and water inputs (Singh *et al.,* 2008). Importantly, the expansion of jatropha cultivation does not endanger existing arable land (Sanjid*et al.,* 2014).

Coupled with deforestation and other environmental degradation, there is an urgent need for conservation of genetic resources of fauna and flora. The rate at which wildlife population dwindle is very alarming. This is as a result of human invasion to natural habitat. Humans have destroyed thousand species, and many passed into oblivion. It has been estimated that 99 percent of recent extinction and currently threatened species have been or will be destroyed by human activities(Dublin, 2023).

Natural regeneration of most plantsis slow and less productive, making it unsuitable for meeting the demand for planting materials (Tolera and Shimelis, 2016). Therefore, a reliable and rapid propagation method is required to address this limitation. Micropropagation offers an alternative approach for large-scale tree propagation and commercial production. Given the pressing issues of deforestation and environmental degradation, conservation of genetic resources of fauna and flora is of utmost importance.

Conservation of trees is vital to safeguard biodiversity, support food security, and provide essential resources for human livelihoods. *Jatropha curcas*, a valuable tree species, holds potential for bio-diesel production and medicinal applications. Micropropagation techniques offer a promising solution for mass production and conservation efforts, crucial in the face of deforestation and the ongoing loss of wildlife species. Through biotechnological advancements, a sustainable biodiversity conservation and economic viability can be achieved. The study conducted at the University of Maiduguri Biotechnology Center sheds light on the in vitro propagation of *Jatropha curcas*, contributing valuable insights to the field of plant propagation and conservation.

2.0 MATERIALS AND METHODS

2.1 Plant Materials and Explant Preparations

Shoot tips of *J. curcas*, were excised from healthy matured trees growing within Maiduguri. The explants collected were washed thoroughly under warm running tap water for 5 min, followed by treatment with 70% ethanol for 10 seconds and was washed thoroughly with double distilled water. Furthermore, surface sterilization was carried out by dipping the explants in 10% and 5% commercial bleach solution (sodium hypochlorite) containing drop of Tween 20 for 10 and 5 minutes respectively, followed by dipping in an antioxidant solution containing 100mgL^{-1}ascorbic acid and 150 mgL^{-1} of citric acid for 10 min to avoid phenolic compound exudation during explants culture, followed by 3 – 4 times rinses in sterilized double distilled water to remove the traces of sterilants.

2.2 Culture Medium Preparation and Conditions

Murashige and Skoog basal medium (Murashige and Skoog, 1962) supplemented with various growth regulators (Auxins and Cytokinins) were used during this study. The steps for the preparation of 1 liter MS medium were used in the present study for induction and proliferation of shoots and roots.

2.2.1 Preparation of Stock Solutions

Stock 1 (Major Salts) Composition

- Calcium chloride (8.8g$L^{-1)}$
- Ammonium nitrate (33gL^{-1})
- Potassium nitrate (38gL^{-1})
- Magnesium sulphate (7.4gL^{-1})
- Potassium phosphate (3.4gL^{-1})

Stock 1 Solutions is a combination of major salt/macro nutrients (Chemical elements required by plant in relatively large amount for their optimum growth). The procedure for preparation of 1 liter MS stock solution was followed to prepare 1000ml MS stock 1 solution at 20(x) concentration. Following MS standard, the appropriate quantity of each of the above listed

salts were measured and weighedon an analytical balance, and were dissolved in 1000ml of distilled water to make 1000ml of the major salt solution.

Stock 2 (Minor Salts) Compositions

- Boric acid ($1.24gL^{-1}$)
- Manganese sulphate ($4.46gL^{-1}$)
- Zinc sulphate ($1.72gL^{-1}$)
- Sodium molybdate ($0.05gL^{-1}$)
- Cupric sulphate ($0.005gL^{-1}$)
- Cobalt chloride ($0.005gL^{-1}$)

Stock 2 solutions is a combination of minor salt/micronutrients (Essential elements which plants needs in relatively small amount). Like stock 1 solution, the procedure for preparation of 1 liter MS stock solution were followed to prepare 1000ml MS stock 2 solutions at 200(x) concentration. Following MS standard, the appropriate grams of each of the above listed salts were measured and weighed on an analytical balance, and were dissolved in 1000ml of distilled water to make 1000ml of the minor salt solution.

Stock 3 (Iron/EDTA) Compositions

- Ferrous sulphate ($5.56gL^{-1}$)
- Ethylenediaminetetracetic acid ($7.46gL^{-1}$)

Stock 3 solutions is a combination of iron and EDTA, 1000ml MS stock 3 solution was prepared at 200(x) concentration. Following MS standard, the appropriate grams of each of the above listed salts were measured and weighed on an analytical balance, and were dissolved in 1000ml of distilled water to make 1000ml of the iron/EDTA solution.

Stock 4 (vitamins) Compositions

i. Thiamine ($0.02gL^{-1}$)
ii. Nacine($0.1gL^{-1}$)
iii. Glycine($0.4gL^{-1}$)
iv. Pyriodixine($0.1gL^{-1}$)

For stock 4 solution, like stock 1, 2 and 3 solution the required grams for MS standard for the preparation of vitamin stock were weighed on an analytical balance and dissolved in 1000ml of distilled water, to make 200(x) concentration of vitamin solution.

2.2.2 Preparation of hormone stock

In the present study three types of plant growth regulators (hormones) were used. They are:

i. Benzyl adenine ($0.2gL^{-1}$)
ii. Indole-3-butiric acid ($0.2gL^{-1}$)
iii. Naphthalene acetic acid($0.2gL^{-1}$)

2.2.2.1 Procedure for Preparation

Benzyl adenine

A stock solution containing 1mg per 1ml of benzyl adenine was prepared. This was achieved by weighing 0.2 gram of BA on an analytical balance, a few drops of 1N NaOH (solvent) were added (to the solute) in order to dissolve well, followed by a distilled water to make 200ml of the solution.

Indole-3-butiric acid

A stock solution containing 1mg per 1ml of indole-3-butiric acid was prepared. This was achieved by weighing 0.2 gram of IBA on an analytical balance, a few drops of Alcohol (solvent) were added (to the solute) in orderto dissolve well, followed by a distilled water to make 200ml of the solution.

Naphthalene acetic acid

A stock solution containing 1mg per 1ml of Naphthalene acetic acid was prepared. This was achieved by weighing 0.2 gram of NAA on an analytical balance, a few drops of 1N NaOH (solvent) were added (to the solute) in orderto dissolve well, followed by a distilled water to make 200ml of the solution.

2.2.3 Preparation of Working Solution

A working solution was prepared from the stock solution, to meet up the appropriate concentration needed for this study. For stock 1 solution 1/20 dilution was prepared and for stock 2, 3, and 4 1/200 dilution was prepared to make 1(x).

2.2.4 MS Medium Composition for 1 liter

i. MS major salt
ii. MS minor Salt
iii. MS vitamins
iv. Myoinositol
v. Iron/EDTA
vi. Agar
vii. Sucrose

All the stock components of the medium were mixed in an appropriate quantity. The final volume of the solution was achieved by the addition of distilled water. The pH of the medium was adjusted to 5.7 with 1 mmol NaOH (Sodium hydroxide) or 1 mmol HCl (Hydrochloric acid). Furthermore, 3% (w/v) sucrose and 0.7% (w/v) agar (Oxoid, Hampshire, England) were added and the medium was heated till boiling to melt agar in a microwave. Glass wares, culture media and instruments were sterilized using oven at 200^{o}C for 1 hour. The medium were then poured in labeled sterilized culture vessels (150 x 25 mm), each containing 20 ml of medium and were autoclave at 120^{o}Cfor 15 minutes. After autoclaving the media were left in the refrigerator to solidify before the explant was transfer.

Culture Procedure

In vitro cultures were maintained in a growth chamber, at 26 ± 2^{o}C with an irradiance of 50µ mol m^{-2} s^{-1} provided by cool white fluorescent tubes (40 w, Philips) for 16 hours photoperiod.

2.3 Shoot Induction and Multiplication

The explants were placed on MS medium augmented with auxin viz. Indole-3-acetic acid (IBA), and cytokinin viz. Benzyl adenine (BA) singly and in combination at different concentrations (0.5 mg/L – 2.5 mg/L). All treatments consist of 3 replicates. The frequency of explants producing shoots, number of shoots per explants and shoot length were recorded at the end of every week for 4 weeks of culture. The plantlets generated were photographed

2.4 In vitro Rooting

The elongated shoot with few leaves were excised in vitro from the shooting medium and transferred to arooting medium containing half strength MS medium supplemented with 2% (w/v) sucroseand plant growth regulators, NAA (Naphthalene acetic acid) and IBA (Indole-3-buteric acid) at different concentration (0.5 mg/L – 2.5 mg/L).The plantlets werecultured and observedfor rooting at interval of one week for four weeks.

2.5 Histological studies

Histological study was carried out on *J. curcas*plantlets after the failed rooting. Samples of two cuttings, the plantlets and the newly harvested explants (control) were taken at random. The basal portion was used for anatomical studies. The section of the samples of about few microns in thickness was prepared using a sharp blade. The sections were stained by the Safranine-Pecro-Amilye-Blue Method (Johansen, 1940), and were microscopically examined and photographed.

2.6 Data Analysis

The data of different treatment were quantified and subjected to statistical analysis using Two Way Analysis of Variance (ANOVA), and Duncan's multiple range tests ($P<0.05$) was employed to determine the significant differences among means of the recorded parameters

3.0 RESULTS AND DISCUSSION

3.1 Results

Theresponse of shoot tip explants of *Jatrophacurcas*to various concentrations and combination of BA and IBA in MS culture medium was studied. Shoot bud differentiation and leaf protrusions was observed and recorded after every week for four weeks as shown in Table 1. The result shows the growth response on a MS culture medium supplemented with benzyl adenine and indole-3-buteric acid across 4 weeks of cultured. The data recorded with respect to number of shoots, number of leaves and shoot length produced at first two weeks of culture in all the treatment shows a rapid response, with little or no response in the last two weeks. Almost all explants across all treatments attended their maximum growth at second week of culture. Moreover, the result also indicated that there were no differences between all the parameters measured across the weeks. Similarly, the result indicates that BA in combination with low concentration of IBA produced the best response compared to any other treatment after four weeks of culture, as it is shown in a combination, MS + BA (0.5) + IBA (0.2) with 2.000 shoot buds with 1.567 cm shoot length as shown in table 2. Treatment with the combination MS + BA (1.0) + IBA (0.4) showedthe least response with 0.667cm short length.

Table 1: Mean growth of *Jatropha curcas* explants at different weeks of culture in MS medium supplemented with BA and IBA.

TREATMENTS		WEEK 1	WEEK 2	WEEK 3	WEEK4
		No. of shoots			
MS		1.000±0.577[a]	1.333±0.667[a]	1.667±0.882[a]	1.667±0.882[a]
MS+BA	(0.5)	2.000±0.336[a]	2.000±0.000[a]	2.000±0.000[a]	2.000±0.000[a]
	(1.0)	1.000±0.000[a]	1.000±0.000[a]	2.000±0.000[a]	2.000±0.000[a]
	(1.5)	1.333±0.333[a]	1.667±0.333[a]	1.667±0.333[a]	1.667±0.333[a]
	(2.0)	1.000±0.577[a]	1.000±0.577[a]	1.000±0.577[a]	1.000±0.577[a]

	(2.5)	1.333±0.667 [a]	1.333±0.667 [a]	1.333±0.667 [a]	1.333±0.667 [a]
MS+BA+IBA	(0.5+0.2)	1.333±0.333 [a]	1.333±0.333 [a]	1.667±0.333 [a]	2.000±0.000 [a]
	(1.0+0.4)	1.000±0.000 [a]	1.000±0.000 [a]	1.000±0.000 [a]	1.000±0.000 [a]
	(1.5+0.6)	0.667±0.333 [a]	0.667±0.333 [a]	0.667±0.333 [a]	1.000±0.577 [a]
	(2.0+0.8)	1.000±0.000 [a]	1.333±0.333 [a]	1.333±0.333 [a]	1.333±0.333 [a]
	(2.5+1.0)	1.333±0.333 [a]	1.333±0.333 [a]	1.333±0.333 [a]	2.000±0.577 [a]
		No. of leaves			
MS		1.000±0.577 [a]	1.667±0.882 [a]	1.667±0.882 [a]	1.500±0.359 [a]
MS+BA	(0.5)	2.000±0.000 [a]	2.000±0.000 [a]	2.000±0.000 [a]	2.000±0.000 [a]
	(1.0)	1.000±0.000 [a]	2.000±0.000 [a]	2.000±0.000 [a]	2.000±0.000 [a]
	(1.5)	1.333±0.333 [a]	1.667±0.333 [a]	1.667±0.333 [a]	1.667±0.333 [a]
	(2.0)	1.000±0.577 [a]	1.000±0.577 [a]	1.000±0.577 [a]	1.000±0.577 [a]
	(2.5)	1.333±0.667 [a]	1.333±0.667 [a]	1.333±0.667 [a]	1.333±0.667 [a]
MS+BA+IBA	(0.5+0.2)	1.333±0.667 [a]	1.667±0.333 [a]	1.667±0.333 [a]	2.000±0.000 [a]
	(1.0+0.4)	1.000±0.000 [a]	1.000±0.000 [a]	1.000±0.000 [a]	1.000±0.000 [a]
	(1.5+0.6)	0.667±0.333 [a]	0.667±0.333 [a]	0.667±0.333 [a]	0.667±0.333 [a]
	(2.0+0.8)	1.000±0.000 [a]	1.333±0.333 [a]	1.333±0.333 [a]	1.333±0.333 [a]
	(2.5+1.0)	1.000±0.000 [a]	1.333±0.333 [a]	1.333±0.333 [a]	1.333±0.333 [a]
		Shoot length			
MS		0.300±0.173 [a]	0.633±0.348 [a]	0.633±0.348 [a]	0.633±0.348 [a]
MS+BA	(0.5)	1.067±0.120[a]	1.333±0.088[a,b]	1.400±0.058[b]	1.467±0.882[b]
	(1.0)	0.567±0.067 [a]	0.867±0.318 [a]	0.933±0.333 [a]	0.933±0.333 [a]
	(1.5)	0.400±0.152 [a]	0.800±0.306 [a]	1.133±0.348 [a]	1.133±0.348 [a]
	(2.0)	0.433±0.338 [a]	0.600±0.200 [a]	0633±0.536 [a]	0.667±0.524 [a]
	(2.5)	0.400±0.306 [a]	0.633±0.376 [a]	0.933±0.498 [a]	0.967±0.524 [a]
MS+BA+IBA	(0.5+0.2)	0.467±0.677 [a]	1.400±0.608 [a]	1.467±0.418 [a]	1.567±0.612 [a]
	(1.0+0.4)	0.333±0.120 [a]	0.667±0.145 [a]	0.667±0.145 [a]	0.667±0.088 [a]
	(1.5+0.6)	0.333±0.203 [a]	0.800±0.404 [a]	0.833±0.600 [a]	0.900±0.458 [a]
	(2.0+0.8)	0.300±0.153[a]	0.800±0.173[a,b]	1.233±0.376 [b]	1.367±0.318 [b]
	(2.5+1.0)	0.300±0.100 [a]	0.433±0.285 [a]	1.000±0.503 [a]	1.167±0.470 [a]

Means with the same superscript in the same row are statistically not significantdifferent from each other.
MS = Murashige and Skoog
BA = Benzyle Adenine
IBA = Indole-3-Buteric acid

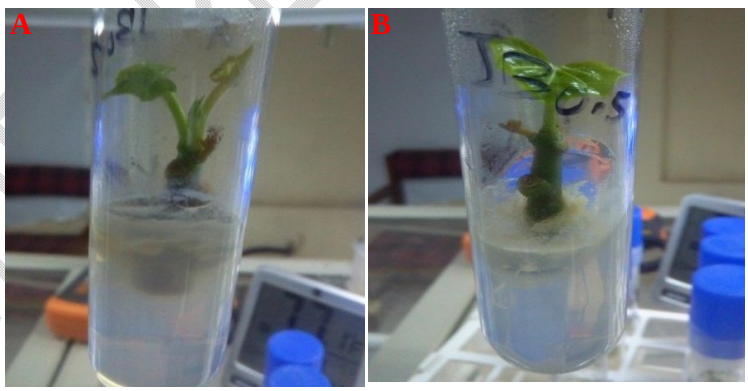

Figure 1: A. *J. curcas* culture in media combination BA0.5mg/l and IBA 0.2mg/l at 4th week. B. *J. curcas* culture in media combination BA0.5mg/l at 4th week.

Table 2: Effect of different concentration of Benzyle Adenine (BA) and Indole-3-Buteric acid (IBA) on explant multiplication by *Jatropha curcas*, at fourth week of culture.

TREATMENTS	NO. OF SHOOT	NO. OF LEAVES	SHOOT LENGTH

MS		1.667±0.882 [a]	1.667±0.882 [a]	0.633±0.348 [a]
MS+BA	(0.5)	2.000±0.000 [a]	2.000±0.000 [a]	1.467±0.888 [a]
	(1.0)	2.000±0.000 [a]	2.000±0.000 [a]	0.933±0.333 [a]
	(1.5)	1.667±0.333 [a]	1.667±0.333 [a]	1.133±0.348 [a]
	(2.0)	1.000±0.577 [a]	1.000±0.577 [a]	0.667±0.524 [a]
	(2.5)	1.333±0.667 [a]	1.333±0.667 [a]	0.967±0.524 [a]
MS+BA+IBA	(0.5+0.2)	2.000±0.000 [a]	2.000±0.000 [a]	1.567±0.612 [a]
	(1.0+0.4)	1.000±0.000 [a]	1.000±0.000 [a]	0.667±0.088 [a]
	(1.5+0.6)	1.000±0.577 [a]	0.667±0.333 [a]	0.900±0.458 [a]
	(2.0+0.8)	1.333±0.333 [a]	1.333±0.333 [a]	1.367±0.317 [a]
	(2.5+1.0)	2.000±0.577 [a]	1.333±0.333 [a]	1.167±0.470 [a]

Means with the same superscript in the same column are statistically not significantly different from each other

MS = Murashige and Skoog
BA = Benzyle Adenine
IBA = Indole-3-Buteric acid

3.2 Discussion

The present study revealedthe establishment of cultures from shot tips obtainedfrom *Jatropha curcas*, on MS full strength media, which is frequently applied in micropropagation of woody species as confirmed by other studies (Sudhersan *et al.*, 2003; Ibrahim, 2008). The explant employed for the study was said to have high rates of cell division, and concentrate the required growth-regulating substances including auxins and cytokinins as reported by Akin-Idowu *et al.*(2009). The explants of *Jatropha curcas* on an MS media supplemented with low level of BA (0.5mg/l) and IBA (0.2mg/l) gave the best results after 30 days of culture;increasing this concentration reduced the shoot length. BA in combination with IBA is considered to be the best plant regulator for achieving maximum growth of *Jatropha curcas* explants. However, some other workers have reported a combination of cytokinin and auxin to be fruitful for axillary and bud sprouting (Dhar and Joshi, 2005).

The response of *Jatropha curcas* explants on the various treated media was rapid at the first 2 weeks of culture where almost all explants attended their maximum growth. It was obvious that some substance appearing to be exudates of phenol clouded the base of the explants, browning the explants mostly in a media with a high concentration of plant growth regulators. These in turn slowedits growth as recorded at 4th week of culture. A phenolic compound exuded as reported by Arnaldos*et al.*(2001), affects in-vitro development negatively and excess exudates of these substances turn the media black and the explant brown. The explants show 92.33% survival rate except for a few cultures attacked by fungal infection.

After regeneration of shoots, the plantlets incubation for rhizogenesis was done in half MS medium (2% sucrose) supplemented with various concentration of Auxins (NAA 0.5 – 2.5 and IBA 0.5 – 2.5mg/l). A similar study conducted by (Maharana*et al.*, 2012) revealedthat half strength MS medium provided better response for rhizogenesis both in terms of rooting frequency and root per shoot. Consequently, other studies also indicated the role of auxin as causative agents for root differentiation (Sarika and Meenakshi, 2008).

During the present study, it was obvious that the plantlets failed to induce rooting in all the media concentration, but instead showed a continuous response on shoot multiplication. The failed rooting might be as a result of callusing as it appeared at the base of the explants. The

development of basal callus is one of the physiological disorders that affect rooting competence of micro plants. There are studies showing the sink effects of these basal callus as it interfered with many physiological processes by trapping essentials growth constituents such as plants growth regulators (Bairu*et al.,* 2011).The result agreed with the study conducted by Shrivastava and Banerjee (2008), on a failed rooting observed on half MS media with plant growth regulators NAA (1.0 – 4.0 mg/l) and IBA (1.0 – 3.0 mg/l). Similar results on the failure of root formation were also reported on other woody plants(Sudhersan and Hussain,2003; Rout *etal.,* 2008; Dhabhai*et al.,* 2010). Other studies reported success in the use of IBA and NAA in root formation as employed in this study. Mayuree and Sompong (2009)study on in vitro shoot multiplication of *Jatropha curcas*revealedthe establishment of root in a media containing 0.5 mg/l IBA.

A further study was conducted after the failed rooting to check the organization of the tissues. The histology of the plantlets and newly harvested explant was observed and compared to detect any irregularities that might have occurred due to the tissue culture protocols employed during the study, and might in turn prevent the plantlet from rooting. The detail microscopic study of the tissues reveals that the vascular system of plantlets and the newly excised explant section, shows similar organization and structures where all the meristematic cells including the pericycle, vascular cambium and phloem parenchyma were present. A similar study on histology conducted by MedzaMve*et al.,* (2013) revealedthat plant regeneration occurs through multiplication of the pre-existing meristem. Another study on histology reveals that, cell proliferation forming a small protrusion originated from the cambial and phloem parenchyma cells below the cork cells developed into adventitious roots (Sawarsan, *et al.,* 2012).

CONCLUSION

On the basis of result of this study, a micropropagation protocol for shoot induction and proliferation has been established. Based on the findings, it may be concluded that BA in combination with IBA in little concentration is considered the best hormone for the in vitro shoot regeneration from the shoot tip explant of *Jatropha curcas.* To address the challenge of achieving root proliferation, it is recommended to reevaluate the hormone combinations used with alternative growth regulators and maintain a controlled microenvironment. By employing these, a more comprehensive and efficient tissue culture protocol can be developed to support the successful regeneration of both shoots and roots in *Jatropha curcas.*

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